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I, KIM MARSHALL, MANAGER EXAMINATION SUPPORT AND SALES, hereby certify that the annexed is a true copy of the Provisional specification in connection with Application No. PP 4723 for a patent by THE UNIVERSITY OF SYDNEY filed on 17 July 1998.



WITNESS my hand this Fourth  
day of August 1999

KIM MARSHALL  
MANAGER EXAMINATION SUPPORT AND  
SALES

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**PROVISIONAL SPECIFICATION**

**Applicants:**

THE UNIVERSITY OF SYDNEY

**Invention Title:**

PROTEASE SUSCEPTIBILITY II

The invention is described in the following statement:

## PROTEASE SUSCEPTIBILITY II

### TECHNICAL FIELD

The present invention relates to: manipulation of the amino acid sequence of tropoelastin, particularly human  
5 tropoelastin, to modify its protease susceptibility; to tropoelastin derivatives having modified protease susceptibility; to peptidomimetic molecules which contain amino acid sequences which correspond to or incorporate the protease susceptible sequences of tropoelastin; and to  
10 uses of the tropoelastin derivatives and peptidomimetic molecules.

The invention also relates to nucleic acid molecules and genetic constructs encoding the amino acid sequences of the derivatives and peptidomimetic molecules of the  
15 invention.

### BACKGROUND ART

The insoluble cross-linked elastin molecule is highly resistant to proteolytic degradation by many proteases. However, tropoelastin, the soluble precursor of elastin,  
20 is far more vulnerable to proteolysis. Attempts at purifying tropoelastin from tissues usually result in a collection of degraded products. This degradation can be decreased by using traditional inhibitors of serine proteases (Franzblau et al., 1989; Rucker, 1982, Rich and  
25 Foster, 1984; Sandberg and Wolt, 1982). Specific degradation has also been noted in cell culture of smooth muscle cells which was attributed to metal proteinases (Hayashi et al., 1995). Even highly purified tropoelastin can degrade into discrete bands on prolonged storage.  
30 This observation has led to an hypothesis that mammalian tropoelastin is occasionally co-purified with an intrinsic protease which will promote its gradual breakdown (Mecham et al., 1976; Mecham et al., 1977; Mecham and Foster, 1977). Experiments have shown that mammalian serum  
35 contains proteases which are capable of degrading

tropoelastin (Romero et al., 1986). Thus, any newly-synthesized unprotected tropoelastin exposed to blood, such as in a blood vessel wall, would be rapidly degraded. Serum has also been shown to induce elastase activity in  
5 smooth muscle cells leading to degradation of elastin (Kobayashi et al., 1994). Elastin peptides are known to be chemotactic and this could be a role of tropoelastin proteolysis *in vivo* (Grosso and Scott, 1993; Bisaccia et al., 1994). However, proteolysis could also result in  
10 inadequate or faulty elastin fiber repair at the site of injury. Serine protease inhibitors have been shown to reduce the degradation of tropoelastin caused by serum (Romero, et al., 1986). These experiments suggested that kallikrein was a candidate serum protease. Other  
15 experiments (McGowan et al., 1996) proposed that plasmin was a major protease involved. Thrombin has been used to digest heterogeneous porcine tropoelastin *in vitro* (Torres et al., 1976). However, none of these studies has provided indication of where the tropoelastin molecule is  
20 cut by proteases.

#### **DESCRIPTION OF THE INVENTION**

In purifying a defined species of recombinant human tropoelastin (Martin et al., 1995) from its fusion partner  
25 the present inventor observed limited and reproducible cleavage of the tropoelastin, by thrombin. The pattern of degradation as seen on SDS-polyacrylamide gels was similar to that seen by others during purification and storage (Mecham et al., 1977). The present inventor recognised  
30 the possibility that this may be because certain portions of tropoelastin are more susceptible to protease action or are more readily available to proteases because of tropoelastin's conformation in solution. A comparison of the sizes of the protease cleavage products with the amino  
35 acid sequence of tropoelastin and the consensus cleavage

sites for the proteases being examined revealed that of the many sites in the tropoelastin amino acid sequence which are homologous to consensus sequences for particular proteases, few were readily digested by proteases. By mapping the sites at which digestion was taking place, susceptible regions were identified thus providing the first precise mapping of protease cleavage sites within any tropoelastin.

From the determination of these susceptible regions, tropoelastin amino acid sequences in these susceptible regions can be modified thus providing reduced tropoelastin derivatives which have a reduced or eliminated protease susceptibility under particular conditions, as compared with the protease susceptibility of tropoelastin under the same conditions.

In the specification and claims, "reduced tropoelastin derivative" means a molecule having a modification of an amino acid sequence in a susceptible region of tropoelastin, which molecule is folded in a functional conformation. "Functional conformation" is defined below. The modification of the amino acid sequence in the susceptible region causes reduced or eliminated protease susceptibility. Reduced tropoelastin derivatives may correspond to full length tropoelastin molecules, single domains of tropoelastin which are encoded by specific exons of the tropoelastin gene or peptides which are encoded by all or part of two neighbouring exons of the tropoelastin gene.

Reduced tropoelastin derivatives may be produced by mutation events including for example, single point mutation in a nucleotide sequence which cause a residue substitution in an amino acid sequence in a susceptible region, or mutation events in a nucleotide sequence which cause an amino acid insertion or deletion in an amino acid sequence in a susceptible region. Reduced tropoelastin derivatives can also be produced by mutation of

tropoelastin sequences, in regions of the tropoelastin molecule which are susceptible to protease digestion, and further mutation in other regions of tropoelastin. The further mutations may or may not alter the susceptibility of the reduced tropoelastin derivative to proteases. Reduced tropoelastin derivatives which contain these mutations may be produced synthetically.

Reduced tropoelastin derivatives may alternatively be produced by chemical modification of amino acid side chains in the derivative which chemically modifies a susceptible region.

Reduced tropoelastin derivatives may in another alternative be produced by protease digestion. Thus according to the invention, a protease digestion product of tropoelastin, which, as a result of digestion, has lost an amino acid sequence which is in a susceptible region, is a reduced tropoelastin derivative.

Reduced tropoelastin derivatives can also be produced by modification of tropoelastin variant amino acid sequences, in regions of the tropoelastin molecule which are susceptible to protease digestion.

In the specification and claims, "variants of tropoelastin" or "tropoelastin variants" means molecules which retain one or more properties of the corresponding tropoelastin molecule, for example, elastin-like properties or macro-molecular binding properties. Elastin-like properties include the phenomenon of recoil after molecular distention and the ability to undergo cross -linking and coacervation. Macro-molecular binding properties include the ability to interact with other macro-molecules, for example glycosylaminoglycans. Tropoelastin variants have an amino acid sequence which is homologous to all or part of the amino acid sequence of a tropoelastin splice form. For the purposes of this description, "homology" between the amino acid sequence of a particular variant and all or part of a tropoelastin

splice form connotes a likeness short of identity, indicative of a derivation of one sequence from the other. In particular, an amino acid sequence is homologous to all or part of a tropoelastin sequence if the alignment of  
5 that amino acid sequence with the relevant tropoelastin sequence reveals an identity of about 65% over any 20 amino acid stretch or over any repetitive element of the molecules shorter than 20 amino acids in length. Such a sequence comparison can be performed via known algorithms  
10 such as that of Lipman and Pearson (1985). Tropoelastin variants may contain amino acid sequence differences as compared with tropoelastin, at a region susceptible to proteolysis, which differences do not alter the protease susceptibility of the tropoelastin variant as compared  
15 with tropoelastin. An example of such an amino acid sequence difference at a susceptible region in a tropoelastin variant may be a conservative amino acid substitution.

Thus reduced tropoelastin derivatives may be produced  
20 by mutation of a tropoelastin variant amino acid sequence, including for example, single point mutations in a nucleotide sequence which causes a residue substitution in an amino acid sequence in a susceptible region of tropoelastin. The reduced tropoelastin derivatives may  
25 also be produced by mutation of a tropoelastin variant amino acid sequence, including for example mutation events in a nucleotide sequence which cause an amino acid insertion or deletion in an amino acid sequence in a susceptible region of tropoelastin. Reduced tropoelastin  
30 derivatives can be produced by mutation of tropoelastin variant sequences, in regions of the tropoelastin molecule which are susceptible to protease digestion, and further mutation in other regions of the reduced tropoelastin variant. The further mutations may or may not alter the  
35 susceptibility of the reduced tropoelastin derivative to proteases. Reduced tropoelastin derivatives which are

produced by the mutation of a tropoelastin variant may be produced synthetically.

Alternatively, reduced tropoelastin derivatives may be produced by chemical modification of amino acid side chains in the derivative which chemically modifies a susceptible region.

Alternatively, reduced tropoelastin derivatives may also be produced by protease digestion of a tropoelastin variant. Thus according to the invention, a protease digestion product of a tropoelastin variant, which, as a result of digestion, has lost an amino acid sequence in a susceptible region, is a reduced tropoelastin derivative.

It is known that tropoelastin genes in nature are expressed as multiple transcripts which are distinguished by alternative splicing of the mRNA as described in, for instance, Indik et al (1990); Oliver et al (1987); Heim et al (1991); Raju et al (1987) and Yeh et al (1987). The methods of the present invention can also be applied to the different splice forms of tropoelastin. The skilled addressee will readily recognise that in applying the methods of the invention to various splice forms of tropoelastin, account must be taken of the presence or absence of the identified cleavage sites in the amino acid sequence of the particular splice form in question.

Human tropoelastins are described by Indik et al (1990) and Tassabehji et al (1997). Bressan et al (1987) describe the amino acid sequence of chick tropoelastin, while Raju et al (1987) describe the amino acid sequence of bovine tropoelastin and Pierce et al (1992) describe the amino acid sequence of rat tropoelastin. Again taking account of variations in amino acid sequence and the existence of different splice forms, the skilled addressee will recognise that the methods of the invention can be applied to tropoelastins from other species.

In a first aspect the present invention provides a method for reducing or eliminating the susceptibility of a



tropoelastin or tropoelastin variant amino acid sequence to proteolysis which method comprises mutating at least one sub-sequence in the tropoelastin or tropoelastin variant amino acid sequence, to reduce or eliminate the susceptibility of the tropoelastin or tropoelastin variant to proteolysis.

In the specification and claims, a "sub-sequence" means a sequence which is cleaved by a protease when tropoelastin or a tropoelastin variant is folded in a functional conformation. A "functional conformation" is the conformation which imparts the elastin -like properties and macro -molecular binding properties to tropoelastin. The sub-sequences correspond to the amino acid sequences in the regions of tropoelastin which are susceptible to proteolysis.

Typically, the mutation involves altering at least one or two residues in the sub-sequence. More preferably, at least one sub-sequence is mutated. More preferably the tropoelastin is human tropoelastin.

In one embodiment, the sub-sequence is digested by a serine protease. Preferably the serine protease is kallikrein. It will be recognised that mutation to remove one or more sub-sequences which are digested by serine protease is of particular benefit when the tropoelastin or tropoelastin variant is to be exposed to serum since the major proteolytic activity of serum for tropoelastin is serine protease activity.

In another embodiment the sub-sequence is digested by a metalloproteinase. Examples of metalloproteinases include gelatinases A and B, the 72kD and 92kD proteases, and MME. The present inventor has noted that cleavage of SHEL and SHEL826A with metalloproteinases leads to reproducible patterns with apparently preferred cleavage sites, evidenced using methods similar to those described here. Significantly SDS-PAGE indicates that cleavage is, at least in some obvious instances, different to the

recognition sequences seen with serine proteases as described in Table 1. Using the 92 kDa metalloproteinase, a characteristic banding pattern was obtained with clear evidence of preferred, more intense bands. For example, 5 using methods described for the serine proteases, N-terminal sequencing of an approximately 10 kDa band derived from SHEL revealed the sequence: LAAAKAAKYGAA. Its location in SHEL is illustrated in Figure 2. Thus a preferred recognition site resides between A and L, which 10 is N-terminally upstream of the identified sequence of this fragment. It will be recognised that mutation to the tropoelastin or a tropoelastin variant sequence to remove one or more sub-sequences which are digested by metalloproteinases is of particular benefit when the 15 tropoelastin or tropoelastin variant is to be exposed to, for example, wound sites, locations of tissue damage and remodelling which can expose the tropoelastin or tropoelastin variant to metalloproteinases.

In a second aspect the present invention provides a 20 reduced tropoelastin derivative exhibiting reduced or eliminated susceptibility to proteolysis in comparison with the corresponding tropoelastin or the corresponding tropoelastin variant, the reduced tropoelastin derivative characterised in that a sub-sequence of the corresponding 25 tropoelastin or corresponding tropoelastin variant amino acid sequences is mutated to eliminate or reduce the susceptibility of the reduced tropoelastin derivative to proteolysis.

Typically, the mutation involves altering at least 30 one or two residues in the sub-sequence. More preferably, at least one sub-sequence is mutated. More preferably the tropoelastin is human tropoelastin.

In one embodiment, the sub-sequence is digested by a serine protease. Preferably the serine protease is 35 kallikrein. In another embodiment the sub-sequence is digested by a metalloproteinase.

Reduced tropoelastin derivatives of the second aspect with mutations appropriate to their use environment can beneficially be used *in vivo* at sites where there is a risk of protease attack on tropoelastin or a variant of tropoelastin, such as in the presence of serum or wound exudate. For instance, the therapeutic use of cross-linked tropoelastin or a cross-linked tropoelastin variant in blood vessel walls would benefit since serum-induced degradation could be reduced. Further, certain modifications should reduce the need to use protease inhibitors during purification of the reduced tropoelastin derivative and result in greater amounts of full-length material if one or more sites are modified to minimise attack by endogenous host proteases.

In a third aspect the present invention provides a method of protecting a tropoelastin or a tropoelastin variant from degradation by serum or a protease selected from the group consisting of kallikrein, thrombin, trypsin and related serine proteases, including elastase, which method comprises mutating at least one sub-sequence in the tropoelastin or tropoelastin variant amino acid sequence to reduce or eliminate the susceptibility of the tropoelastin or tropoelastin variant to proteolysis. Preferably the protease is kallikrein. Preferably the tropoelastin is human tropoelastin.

In a fourth aspect the present invention provides a method of protecting a tropoelastin or a tropoelastin variant from degradation by proteolytic attack, which method comprises mutating at least one sub-sequence in the tropoelastin or tropoelastin variant amino acid sequence to reduce or eliminate the susceptibility of the tropoelastin or tropoelastin variant to proteolysis. In one embodiment the sub-sequence is digested by a metalloproteinase.

As described above, amino acid sequences of non-human tropoelastins have been determined, including the amino

acid sequences of chick tropoelastin, bovine tropoelastin and rat tropoelastin (Bressan et al. 1987, Raju et al. 1987, Pierce et al. 1992). A comparison of these non-human tropoelastin amino acid sequences with tropoelastin  
5 reveals that particular regions of tropoelastin which are susceptible to proteolysis as identified in the present invention are conserved in these non-human tropoelastins. Therefore it is likely that these particular regions in the non-human tropoelastins will be susceptible to  
10 proteolysis.

Using the sub-sequences described in Table 1 and comparing these with the 'nr' database using 'tblastn' at the NCBI Blast facility (<http://www.ncbi.nlm.nih.gov/BLAST>) shows the following:

15

(i) human tropoelastin:

554 VPTGAGVKPKAPGVGGAF 607

bovine tropoelastin, exon 14

20 373 VPTGAGVKPKAPGGGGAF 426

mouse tropoelastin mRNA complete cds

694 VPTGTGVKAKAPGGGGAF 747

25 bovine elastin a mRNA complete cds

545 VPTGAGVKPKAQVGAGAF 598

bovine elastin b mRNA complete cds

545 VPTGAGVKPKAQVGAGAF 598

30

bovine elastin c mRNA complete cds

545 VPTGAGVKPKAQVGAGAF 598

rat tropoelastin mRNA 3' end

35 646 VPTGTGVKAKVPGGGG 693

chicken tropoelastin mRNA complete cds

572 VPTGTGIKAKGPGAG 616

5 (ii) human tropoelastin:

1664 KVAAKAQLRAAAGLGAG 1714

rat tropoelastin mRNA 3' end

1837 KAAAKAQYRAAAGLGAG 1887

10

mouse tropoelastin mRNA complete cds

1795 KAAAKAQYRAAAGLGAG 1845

bovine elastin a mRNA complete cds

15 1649 KAAAKAQFRAAAGLPAG 1699

bovine elastin b mRNA complete cds

1607 KAAAKAQFRAAAGLPAG 1657

20 bovine elastin c mRNA complete cds

1547 KAAAKAQFRAAAGLPAG 1597

which demonstrates that the sequences are highly homologous between species, supporting the proposition that taking account of sequence differences the methods of the invention can be applied to different tropoelastin species.

In the human tropoelastin splice form described in more detail herein and illustrated in Figure 2, the cleavage in serum occurs between residues 516 and 517; 566 and 567; 443 and 444; 505 and 506; and 566 and 567. Thus for this splice form the alteration to the sequence to influence serine protease susceptibility preferably involves modification of at least one of residues 516, 517, 566, 567, 443, 444, 505, 506, 566 and 567. Alterations to reduce susceptibility to protease attack

can be considered to involve removal or modification of the recognition site. An example of this modification is the replacement of lysine or arginine by an amino acid residue that is not positively charged. An example of  
5 this approach is the use of leucine to replace arginine in the sequence R/AAAGLG of Table 1 using common methods of mutagenesis such as those available commercially in kit form.

Reduced tropoelastin derivatives of the invention  
10 include:

SHEL  $\delta$  26a (which is illustrated in Figure 3),  
SHEL $\delta$ mod (which is illustrated, by comparison with SHEL, in Figure 4), the N-terminal 2/3 of the splice form illustrated in Figure 2 and the C-terminal 1/3 of the  
15 splice form illustrated in Figure 2; and the products produced by cleavage of SHEL $\delta$ 26A with kallikrein which results in two dominant species, defined by the cutting site with the sequence R/AAAGLG which corresponds to positions 515/516 (Table 1).

20 As the inventor has determined the regions of tropoelastin which are susceptible to proteolysis, tropoelastin can be modified by inserting sequences which correspond to amino acid sequences in the susceptible regions of tropoelastin, into the tropoelastin amino acid  
25 sequence, thus providing enhanced tropoelastin derivatives which have enhanced protease susceptibility under particular conditions as compared with the protease susceptibility of tropoelastin under the same conditions.

Thus, in the specification and claims, "enhanced  
30 tropoelastin derivative" means a molecule produced by inserting a sequence which corresponds to an amino acid sequence in a susceptible region of tropoelastin, into the tropoelastin amino acid sequence, which molecule is folded in a functional conformation. The insertion of the amino  
35 acid sequence which corresponds to the amino acid sequence

of a susceptible region causes enhanced protease susceptibility. Enhanced tropoelastin derivatives may correspond to full length tropoelastin molecules, single domains of tropoelastin which are encoded by specific  
5 exons of the tropoelastin gene or peptides which are encoded by all or part of two neighbouring exons of the tropoelastin gene.

Insertion of the amino acid sequence into tropoelastin, may occur by, for example, splicing a  
10 peptide which has an amino acid sequence which corresponds to a susceptible region in tropoelastin, into tropoelastin. Thus, enhanced tropoelastin derivatives may be produced by mutation events including a mutation in a nucleotide sequence which causes an insertion of a peptide  
15 in the tropoelastin amino acid sequence wherein the inserted peptide corresponds to an amino acid sequence in a susceptible region of tropoelastin.

Alternatively, insertion of the amino acid sequence into tropoelastin may occur by modifying an amino acid  
20 sequence in a region of tropoelastin, by residue insertion, substitution or deletion, so as to generate an amino acid sequence in that region of tropoelastin which is the same as an amino acid sequence in a susceptible region of tropoelastin. Thus, enhanced tropoelastin  
25 derivatives may be produced by mutation events including a mutation in a nucleotide sequence which causes residue insertion, substitution or deletion in a region of tropoelastin, wherein the mutation events produce at the region, an amino acid sequence which corresponds to a  
30 susceptible region of tropoelastin.

Enhanced tropoelastin derivatives which have an inserted amino acid sequence in accordance with either of the above, may be mutated further by residue insertion, substitution or deletion, or further amino acid sequence  
35 insertion. The further mutations may or may not alter the susceptibility of the enhanced tropoelastin derivative to

proteases. Enhanced tropoelastin derivatives which contain these mutations may be produced synthetically.

Enhanced tropoelastin derivatives can be produced by modification of tropoelastin variant amino acid sequences, in regions of tropoelastin which are susceptible to protease digestion.

Thus, enhanced tropoelastin derivatives may be produced by mutation of a tropoelastin variant amino acid sequence including a mutation in a nucleotide sequence which causes an insertion of a peptide in the tropoelastin variant amino acid sequence wherein the inserted peptide corresponds to an amino acid sequence in a susceptible region of tropoelastin.

Alternatively, enhanced tropoelastin derivatives may be produced by mutation of a tropoelastin variant amino acid sequence including a mutation in a nucleotide sequence which causes residue insertion, substitution or deletion in a region of a tropoelastin variant amino acid sequence, wherein the mutation events produce at the region, an amino acid sequence which corresponds to a susceptible region of tropoelastin.

Enhanced tropoelastin derivatives which have an inserted amino acid sequence in accordance with either of the above, may be mutated further by residue insertion, substitution or deletion, or further amino acid sequence insertion in the tropoelastin variant amino acid sequence. The further mutations may or may not alter the susceptibility of the enhanced tropoelastin derivative to proteases. Enhanced tropoelastin derivatives which contain these mutations may be produced synthetically.

As described above, the tropoelastin amino acid sequence is known to be translated in various mRNA splice forms in humans and non-human animals. Further the comparison of human and non-human tropoelastin amino acid sequences reveals amino acid homology between tropoelastin amino acid sequences. Thus, these various isoforms of



human and non-human tropoelastin and the mRNA splice forms encoding them can be modified to provide the enhanced tropoelastin derivatives of the invention.

5 In a fifth aspect the invention provides a method for enhancing the susceptibility of a tropoelastin or tropoelastin variant amino acid sequence to proteolysis, which method comprises inserting a sub-sequence into a tropoelastin or tropoelastin variant amino acid sequence to enhance the susceptibility of the tropoelastin or  
10 tropoelastin variant to proteolysis. As described above, in the specification and claims, a "sub-sequence" means a sequence which is cleaved by a protease when tropoelastin or a tropoelastin variant is folded in a functional conformation. The sub-sequences correspond to the amino  
15 acid sequences in the regions of tropoelastin which are susceptible to proteolysis. Typically, at least one sub-sequence is inserted into the tropoelastin or tropoelastin variant amino acid sequence. Preferably the tropoelastin is human tropoelastin.

20 In one embodiment the inserted sub-sequence is digested by a protease selected from the group consisting of thrombin, kallikrein, trypsin and related serine proteases, including elastase. In another embodiment the inserted sub-sequence is digested by a metalloproteinase.

25 In a sixth aspect the invention provides an enhanced tropoelastin derivative exhibiting enhanced susceptibility to proteolysis in comparison with the corresponding tropoelastin or tropoelastin variant, the enhanced tropoelastin derivative characterised in that a sub-  
30 sequence is inserted into the tropoelastin or tropoelastin variant amino acid sequence to enhance the susceptibility of the enhanced tropoelastin derivative to proteolysis. Typically, at least one sub-sequence is inserted into the tropoelastin or tropoelastin variant amino acid sequence.  
35 Preferably the tropoelastin is human tropoelastin.

In one embodiment, the sub-sequence may be digested by a serine protease. An example of the incorporation of such a sub-sequence is a polypeptide with a non-contiguous additional copy or cassette of the sequence:

5 KVAAKAQLRAAAGLGAG, at the approximate centre of which resides the R/A bond commonly recognised and cleaved by thrombin and kallikrein and evidenced in serum. Further examples include modifications to the naturally occurring sequences of splice forms of tropoelastin. For example,  
10 in SHEL (illustrated in Figure 2) the sequence encompassing and including residues 678 to 690 is AAKAAKYGAAGLG, which is similar to AAKAQLRAAAGLG, and is altered by site-directed mutagenesis to provide the latter sequence.

15 In another embodiment the sub-sequence is digested by a metalloproteinase.

The enhanced tropoelastin derivative of the sixth aspect can beneficially be used *in vivo* at sites where it is desirable to augment protease attack on the derivative.  
20 Suitable molecules for manipulation include human tropoelastin molecules. In this case, the modified tropoelastin will be of use in situations in which it is desirable to have the tropoelastin or tropoelastin variant degrade rapidly. Such situations include revealing and/or  
25 release of peptides with desirable properties, to accelerate tissue repair.

As the inventor has determined the regions of tropoelastin which are susceptible to proteolysis, the susceptibility of a polypeptide to proteolysis can be  
30 modified by inserting sequences which correspond to amino acid sequences in the susceptible regions of tropoelastin, into the polypeptide amino acid sequence, thus providing polypeptide derivatives which have enhanced protease susceptibility under particular conditions compared with  
35 the same polypeptide which does not contain the said inserted sequence, (the corresponding polypeptide) under

the same conditions.

In the specification and claims "polypeptide derivative" means a polypeptide produced by inserting a sequence which corresponds to an amino acid sequence in a susceptible region of tropoelastin, into the polypeptide sequence. The insertion of the amino acid sequence which corresponds to the amino acid sequence of a susceptible region of tropoelastin into the polypeptide sequence, causes the enhanced protease susceptibility of the polypeptide derivative.

Insertion of the amino acid sequence into the polypeptide sequence may occur by, for example, splicing a peptide which has an amino acid sequence which corresponds to a susceptible region in tropoelastin, into the polypeptide. Thus polypeptide derivatives may be produced by mutation events including a mutation in a nucleotide sequence which causes an insertion of a peptide in the polypeptide amino acid sequence wherein the inserted peptide corresponds to an amino acid sequence in a susceptible region of tropoelastin.

Alternatively, insertion of the amino acid sequence into the polypeptide sequence may occur by modifying an amino acid sequence in the region of the polypeptide, by residue insertion, substitution or deletion, so as to generate an amino acid sequence in that region of the polypeptide which is the same as an amino acid sequence in a susceptible region of tropoelastin. Thus, polypeptide derivatives may be produced by mutation events including a mutation in a nucleotide sequence which causes residue insertion, substitution or deletion in a region of the polypeptide, wherein the mutation events produce at the region, an amino acid sequence which corresponds to a susceptible region of tropoelastin.

Polypeptide derivatives which contain these mutations may be produced synthetically.

Thus in a seventh aspect the invention provides a method for enhancing the susceptibility of a polypeptide amino acid sequence to proteolysis, which method comprises inserting an amino acid sequence which corresponds to an amino acid sequence in a susceptible region of tropoelastin into a polypeptide amino acid sequence to enhance the susceptibility of the polypeptide to proteolysis. Typically at least one sequence corresponding to an amino acid sequence in a susceptible region of tropoelastin is inserted into the polypeptide amino acid sequence.

In one embodiment the inserted sequence is digested by a protease selected from the group consisting of thrombin, kallikrein, trypsin and related serine proteases, including elastase. In another embodiment the inserted sequence is digested by a metalloproteinase.

In an eighth aspect, the invention provides a polypeptide derivative exhibiting enhanced susceptibility to proteolysis in comparison with the corresponding polypeptide, the polypeptide derivative characterised in that an amino acid sequence which corresponds to an amino acid sequence in a susceptible region of tropoelastin is inserted into the polypeptide amino acid sequence to enhance the susceptibility of the polypeptide to proteolysis. Typically at least one sequence corresponding to an amino acid sequence in a susceptible region of tropoelastin is inserted into the polypeptide amino acid sequence.

In one embodiment, the inserted sequence may be digested by a serine protease. Preferably the serine protease is kallikrein. In another embodiment the inserted sequence may be digested by a metalloproteinase.

As the inventor has determined the regions of tropoelastin which are susceptible to proteolysis, these regions can be used to direct the specific release of peptide domains from reduced or enhanced tropoelastin

derivatives of the second and sixth aspects of the invention or the specific release of peptides from the polypeptide derivatives of the eighth aspect of the invention. Typically, amino acid sequences which correspond to the susceptible regions of tropoelastin are inserted between the derivative and the peptide domain thus providing a chimeric derivative which can be digested at the susceptible region by a specific protease to release the peptide domain from the derivative.

10        In the specification and claims, "chimeric derivative" means a molecule produced by linking a derivative selected from the group consisting of a reduced tropoelastin derivative, enhanced tropoelastin derivative and a polypeptide derivative, with a peptide domain via an amino acid sequence which corresponds to an amino acid sequence in a susceptible region of tropoelastin. The amino acid sequence which corresponds to the amino acid sequence of a susceptible region of tropoelastin causes the release of the peptide domain from the derivative when the chimeric derivative is digested by a specific protease.

20        Chimeric derivatives may be produced by recombinant DNA techniques, including for example the construction of a nucleotide sequence which encodes the derivative, the susceptible region and the peptide domain in a single open reading frame. The chimeric derivatives may alternatively be produced synthetically.

25        Thus in a ninth aspect, the invention provides a method for producing a chimeric derivative which method comprises linking a derivative selected from the group consisting of a reduced tropoelastin derivative, enhanced tropoelastin derivative and a polypeptide derivative, with a peptide domain via an amino acid sequence which corresponds to an amino acid sequence in a susceptible region of tropoelastin.

In one embodiment, the amino acid sequence which corresponds to an amino acid sequence in a susceptible region of tropoelastin sequence may be digested by a serine protease. Preferably the serine protease is kallikrein. In another embodiment the sequence may be digested by a metalloproteinase.

In a tenth aspect, the invention provides a chimeric derivative which comprises a derivative selected from the group consisting of a reduced tropoelastin derivative, enhanced tropoelastin derivative and a polypeptide derivative, which is linked with a peptide domain via an amino acid sequence which corresponds to an amino acid sequence in a susceptible region of tropoelastin.

In one embodiment, the amino acid sequence which corresponds to an amino acid sequence in a susceptible region of tropoelastin sequence may be digested by a serine protease. Preferably the serine protease is kallikrein. In another embodiment the sequence may be digested by a metalloproteinase.

The chimeric derivatives of the invention will find application where the peptide domain has a particular biological function, including for example chemotaxis, cell proliferation or cell activation. These biological functions will be effected by digestion of the chimeric derivative at the sub-sequence by a particular protease so as to release the peptide domain from the derivative domain.

The mutations in accordance with this invention may be generated by conventional site-directed or random mutagenesis. Oligonucleotide-directed mutagenesis is a further option. This method comprises:

1. synthesis of an oligonucleotide with a sequence that contains the desired nucleotide substitution (mutation);
2. hybridising the oligonucleotide to a template comprising a structural sequence encoding

tropoelastin; and

3. using a DNA polymerase to extend the oligonucleotide as a primer.

Another approach which is particularly suited to  
5 situations where a synthetic polynucleotide encoding the tropoelastin is prepared from oligonucleotide blocks bounded by restriction sites, is cassette mutagenesis where entire restriction fragments are replaced.

As the inventor has identified regions of  
10 tropoelastin which are susceptible to proteolysis, it is possible to use the amino acid sequences in the susceptible regions to prepare protease inhibitor molecules which are also known as peptidomimetic molecules. In the specification and claims,  
15 "peptidomimetic molecules" means molecules which imitate a region of tropoelastin which is susceptible to proteolysis, and which therefore compete with the susceptible region for the catalytic domain in a protease. Typically the peptidomimetic molecules are peptides or  
20 peptide -like.

The peptidomimetic molecules of the invention may be structurally similar to peptides. They may include an amino acid sequence of a tropoelastin or of a variant of tropoelastin which is or includes a proteolytic site. The  
25 peptidomimetic molecules of the invention may include amino acid residues which are modified at one or more chemical groups and may be linked by non-peptide bonds. These molecules can be used in situations in which it is desirable to prevent the action of the relevant proteases.

30 In an eleventh aspect the present invention provides a peptide or a peptidomimetic molecule including all or part of a peptide selected from the group consisting of KAPGVGGAF, RAAAGLG, RLSPELREGD, KAAQFGLVPGV, KSAAKVAAKAQLRAA, RLSPELRE and LAAAKAAKYGAA.

35 The peptides of this aspect of the invention may be short peptides consisting of all or part of a sequence

selected from the group consisting of KAPGVGGAF, RAAAGLG, RSLSPELREGD, KAAQFGLVPGV, KSAAKVAAKAQLRAA, RSLSPELRE and LAAAKAAKYGAA each in combination with upstream sequence to generate a peptide typically of the order of 15 residues  
5 although it will be understood that in some cases smaller peptides could be used and frequently larger sequences could be used. The peptides can be larger molecules containing one or more of these sequences. In addition structural analogues of these peptides are included within  
10 the scope of peptidomimetic molecules of the invention, and include for instance molecules containing modified amino acid residues.

A preferred molecule is one in which the natural cleavage site would typically be located about the centre  
15 of the peptide or peptidomimetic molecule. An example peptide is H-Ala-Ala-Lys-Ala-Gln-Leu-Arg-Ala-Ala-Ala-Gly-Leu-Gly-Ala-OH which is based on the sequence RAAAGLGA, in its context within the sequence of tropoelastin(s). A peptidomimetic form of this molecule is H-Ala-Ala-Lys-Ala-  
20 Gln-Leu-Arg-R-Ala-Ala-Ala-Gly-Leu-Gly-Ala-OH (where R = a reduced peptide bond).

A further category of molecules contain one or more attached reactive groups for the covalent modification of an interacting protease leading to further inhibition of  
25 activity of the protease. It is recognised that there is a plethora of chemically reactive groups available as biochemical reagents, which are often utilised in the construction of chemical crosslinkers. A subset of these may be found in the Pierce Product Catalog (1997) Chapter  
30 7 pp133 to 154. The reactive group can be placed at the ends or internal to the molecule to provide a proximity to the reacting entity.

The peptides and peptidomimetic molecules of the invention are useful in a number of different environments  
35 including in the purification of tropoelastin, as a pharmaceutical agent which can be provided in an inhalant



form for protecting lung tissue from damage related to elastolytic protease attack on elastin (a major cause of lung damage in smokers) and in any other environment in which competitive inhibition of protease active sites  
5 recognising these peptides is desirable.

In a twelfth aspect the present invention provides a method for enhancing the purification of a tropoelastin or a tropoelastin variant which method comprises including at least one peptide or peptidomimetic molecule of the  
10 eleventh aspect of the invention in the crude tropoelastin or tropoelastin variant preparation which is being subjected to purification.

In a thirteenth aspect the present invention provides a pharmaceutical composition comprising a derivative  
15 selected from the group consisting of a reduced tropoelastin derivative, an enhanced tropoelastin derivative, a polypeptide derivative and a chimeric derivative, or a peptide or peptidomimetic molecule of the invention together with a pharmaceutically acceptable  
20 carrier or diluent. Formulations of the derivatives or peptides or peptidomimetic molecules of the present invention are prepared in accordance with standard pharmaceutical techniques. Preferred formulations in accordance with the invention include inhalant  
25 formulations, incorporation into emulsions designed for localised use, attachment to surfaces such as a stent and injectable formulations. In addition the present inventor recognises that the compositions of the invention can be adapted for use in situations in which it is desirable to  
30 limit protease activity such as that leading to clot formation.

In an fourteenth aspect the present invention provides a nucleotide sequence encoding a derivative selected from the group consisting of a reduced  
35 tropoelastin derivative, an enhanced tropoelastin derivative, a polypeptide derivative and a chimeric

derivative or a peptide or peptidomimetic molecule of the invention.

5 The nucleotide may be provided as a recombinant DNA molecule including vector DNA. Polynucleotides can be prepared using a combination of synthetic and cDNA techniques to form hybrid modified polynucleotide molecules. These molecules also fall within the scope of this invention.

10 Vectors useful in this invention include plasmids, phages and phagemids. The synthetic polynucleotides of the present invention can also be used in integrative expression systems or lytic or comparable expression systems.

15 Suitable vectors will generally contain origins of replication and control sequences which are derived from species compatible with the intended expression host. Typically these vectors include a promoter located upstream from the polynucleotide, together with a ribosome binding site if intended for prokaryotic expression, and a phenotypic selection gene such as one conferring  
20 antibiotic resistance or supplying an auxotrophic requirement. For production vectors, vectors which provide for enhanced stability through partitioning may be chosen. Where integrative vectors are used it is not  
25 necessary for the vector to have an origin of replication. Lytic and other comparable expression systems do not need to have those functions required for maintenance of vectors in hosts.

For *E. coli* typical vectors include pBR322,  
30 pBluescript II SK<sup>+</sup>, pGEX-2T, pTrc99A, pET series vectors, particularly pET3a and pET3d, (Studier et al., 1990) and derivatives of these vectors.

In a fifteenth aspect the present invention provides a cell capable of expressing a nucleotide sequence of the  
35 fourteenth aspect of the invention.

A preferred expression system is an *E. coli*

expression system. However, the invention includes within its scope the use of other hosts capable of expressing protein from the polynucleotides designed for use in *E. coli* as well as to the use of synthetic polynucleotides suitable for use in other expression systems such as other microbial expression systems. These other expression systems include yeast, and bacterial expression systems, insect cell expression systems, and expression systems involving other eukaryotic cell lines or whole organisms.

10        Examples of *E. coli* hosts include *E. coli* B strain derivatives (Studier *et al*, 1990), NM522 (Gough and Murray, 1983) and XL1-Blue (Bullock *et al*, 1987).

         In a sixteenth aspect the present invention provides an expression product of a cell of the fifteenth aspect of the invention encoded by a nucleotide sequence of the  
15        fourteenth aspect of the invention.

         The expression products of the invention may be fused expression products which include all or part of a protein encoded by the vector in peptide linkage with the  
20        expression product. They may also include, for example, an N-terminal methionine or other additional residues which do not permanently impair the elastic properties of the product.

         Typically the fusion is to the N-terminus of the  
25        desired expression product. An example of a suitable protein is glutathione *S*-transferase (Smith and Johnson 1988). The fused protein sequence may be chosen in order to cause the expression product to be secreted or expressed as a cell surface protein to simplify  
30        purification or expressed as a cytoplasmic protein.

         The expressed fusion products may subsequently be treated to remove the fused protein sequences to provide free modified tropoelastin. Treatment is typically through protease treatment, or in the case of secretion removal is  
35        effected by endogenous host secretion machinery. An example of this is secretion by yeasts, including but not

limited to *S. cerevisiae* and *S. pombe*.

Non-fused systems include the introduction of or use of a pre-existing methionine codon. An example of this is the use of pET3a and pET3d in *E. coli*.

5       According to a seventeenth aspect of the present invention there is provided a process for the production of an expression product of the sixteenth aspect comprising:

                  providing a cell of the fifteenth aspect;  
10       culturing it under conditions suitable for the expression of the product of the sixteenth aspect; and collecting the expression product.

                  In a eighteenth aspect the present invention provides an implant formed from one or more derivatives selected  
15       from the group consisting of a reduced tropoelastin derivative, an enhanced tropoelastin derivative, a polypeptide derivative and a chimeric derivative. Where the derivative has reduced proteolytic susceptibility the implant will be intended to be maintained *in situ* over a  
20       considerable period of time whereas when the derivative has enhanced proteolytic susceptibility the implant will be intended to be maintained *in situ* over a short period of time and indeed the rapid dissolution of the implant will be desired such as where it is desired that the  
25       implant is replaced by endogenous connective tissue.

                  Tropoelastin derivatives (ie reduced tropoelastin derivatives and enhanced tropoelastin derivatives) of the invention can be cross-linked to form elastin or elastin-like material or can be cross-linked in conjunction with  
30       other biological or synthetic molecules to form a composite material. The cross-linking of the tropoelastin derivative can be achieved by chemical oxidation of lysine side chains using processes such as ruthenium tetroxide mediated oxidation and quinone mediated oxidation, or by  
35       using bifunctional chemical cross-linking agents such as dithiobis (succinimidylpropionate), dimethyl adipimidate

or dimethyl pimelimidate and those within heterologous sites such as agents that contain UV activated cross-linking domain(s). Another alternative is the cross-linking of lysine and glutamic acid side chains.

5       The tropoelastin derivatives (ie reduced tropoelastin derivatives and enhanced tropoelastin derivatives) may also be enzymatically cross-linked by methods including lysyl oxidase mediated oxidation or be cross-linked using gamma irradiation. The implants are formed into the  
10       required shape by cross-linking the tropoelastin derivative in a mould which conforms to the desired shape of the implant. Where the implant is required to be used in sheet form the derivative can be cross-linked on a flat surface. Relevant methodologies are described in, for  
15       example, US 4 474 851 and US 5 250 516. The elastomeric materials may be exclusively prepared from one or more derivatives or may be composites prepared from one or more derivatives together with other materials.

20

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows a schematic diagram illustrating the relative positions of protease sites identified by N-terminal sequencing for serum, kallikrein and thrombin.  
25       Major sites are indicated with a solid bar while minor sites are indicated with a stippled bar. Since plasmin fragments all contained the same N-terminal sequence the site of cleavage could not be identified unambiguously. The trypsin fragments identified similarly all contained  
30       the same N-terminal sequence. Therefore, the likely regions of cleavage for plasmin and trypsin are not shown.

Figure 2 shows the position of the protease recognition sites in a human tropoelastin (SHEL).

Figure 3 shows the amino acid sequence of SHEL $\delta$ 26A  
35       compared to the amino acid sequence of SHEL.

Figure 4 shows the amino acid sequence of SHEL $\delta$ mod.

Figure 5 shows degradation of SHEL with serum. a. After addition of serum to SHEL for 1,2 ,3 or 18 hours, SHEL is fragmented into a number of distinct bands as seen  
5 by 10% SDS-PAGE. Products from overnight digestion are very similar to products present after one hour of digestion. B. SHEL and SHEL $\delta$ 26A peptide fragments produced by serum digestion and purified by butanol solubilisation were analysed by 10% SDS-PAGE. A band at 15kDa appears  
10 with SHEL $\delta$ 26A (arrow) in place of the 22 and 18kDa bands. Approximate sizes of fragments produced are shown in kDa. Size markers are shown in kDa.

Figure 6 shows the effect of protease inhibitors on serum degradation of SHEL. The presence (+) or absence (-)  
15 of various protease inhibitors on the amount of full-length SHEL was analysed by 8% SDS-PAGE. Full-length SHEL is increased in the presence of Pefabloc SC (0.5mM), Pefabloc PK (50 $\mu$ M) and PMSF (5mM) compared with serum alone, while there is no noticeable effect in the presence of hirudin  
20 (1U). In contrast, the presence of EDTA results in a decrease in the amount of full-length SHEL.

Figure 7 shows the effect of thrombin on SHEL and SHEL $\delta$ 26A. (a) increasing amounts of thrombin were added to SHEL and analysed by 8% SDS-PAGE. Three major degradation  
25 products are seen estimated at 45, 34 and 22kDa as well as a 13kDa fragment not seen in this gel. (b) effect of thrombin (1U) on degradation of SHEL $\delta$ 26A compared with SHEL, analysed by 8% SDS-PAGE. A band at 15kDa (arrow) appears in the place of the 22kDa band. Fragment sizes are  
30 estimated in kDa. Size markers (S) are shown in kDa.

Figure 8 shows the effect of kallikrein on SHEL and SHEL $\delta$ 26A (a) Increasing concentrations of kallikrein were added to SHEL and analysed by 8% SDS-PAGE. Three major fragments are seen at 45, 22 and 18kDa. The 22 kDa

fragment disappears with higher concentrations or longer incubations with kallikrein. (b) Effect of kallikrein ( $6 \times 10^{-4}$ U) on degradation of SHEL $\delta$ 26A compared with SHEL, analysed by 8% SDS-PAGE. Only two fragments are seen with SHEL $\delta$ 26A at 45 and 15kDa (arrow). Fragment sizes and size markers (S) are shown in kDa.

Figure 9 shows the effect of bovine trypsin on SHEL and SHEL $\delta$ 26A. (a) increasing concentrations of bovine trypsin were added to SHEL and analysed by 10% SDS-PAGE. Dilute amounts of trypsin produce prominent bands at 50, 45, 40, 34, 31-255, 22 and 18 kDa, similar to serum produced peptides. Higher concentrations completely degrade SHEL (not shown). (b) Effect of bovine trypsin ( $2 \times 10^{-3}$ U) on SHEL $\delta$ 26A compared with SHEL analysed by 10% SDS-PAGE. The overall pattern of fragments is the same as for SHEL but the size of the smaller fragments are all approximately 4kDa less. Fragment sizes and size markers (S) are shown in kDa.

Figure 10 shows the effect of plasmin on SHEL and SHEL $\delta$ 26A. (a) Increasing concentrations of plasmin were added to SHEL and analysed by 10% SDS-PAGE. Dilute amounts of plasmin produce prominent bands at 50, 45, 40, 34, 28, 22 and 18kDa similar to serum-produced peptides. Higher concentrations of plasmin or longer incubations completely degrade SHEL (not shown). (b) Effect of plasmin ( $7.4 \times 10^{-5}$ U) on SHEL $\delta$ 26A compared with SHEL, analysed by 10% SDS-PAGE. The overall pattern is similar to SHEL but the smaller fragments are approximately 4kDa smaller. Fragment sizes and size markers (S) are shown in kDa.

Figure 11 shows the effect of human leukocyte elastase (HLE) on SHEL and SHEL $\delta$ 26A. Increasing concentrations of HLE were added to SHEL and analysed by 10% SDS-PAGE. Degradation was extensive but prominent sharp fragments were identified at 32 and 18kDa with the other bands being

diffuse. (b) Effect of HLE ( $1.6 \times 10^{-3}$ U) on SHEL $\delta$ 26A compared with SHEL, analysed by 10% SDS-PAGE. A very similar profile to that of SHEL is seen but fragments are uniformly 4 kDa smaller. Fragment sizes and size markers (S) are shown in kDa.

Figure 12 shows the typical effect of S-GAL and SPS-peptide on degradation of SHEL. Addition of SPS-peptide or S-GAL to SHEL reactions containing proteases was examined by 10% SDS-PAGE. The conditions used are serum, 1/2 dilution 20min; trypsin 20min; plasmin  $1.5 \times 10^{-5}$ U 20min; kallikrein  $15 \times 10^{-4}$ U 40min; thrombin 0.1U 20min; HLE 70min. Thrombin and kallikrein were used with a 100:1 ratio. Gels were scanned by densitometry and the relative amount of each full-length SHEL band is shown in a histogram. In each case, the presence of SPS-peptide resulted in more full-length SHEL remaining than in control reactions, while the presence of S-GAL also resulted in more full-length SHEL in the presence of thrombin and HLE only.

Figure 13 shows the effect of coacervation on the degradation of SHEL by proteases. (a) SHEL degradation in the presence of NaCl conducive to coacervation of SHEL at 37°C (-). (b) Control reactions in the presence (+) and absence (-) of NaCl were performed at 16°C. Significant protection from kallikrein and thrombin proteolysis is seen when SHEL is coacervated. Protection is also seen from serum, trypsin and HLE while none is seen with plasmin. At 16°C all of the proteases degraded SHEL to a similar extent in both the presence and absence of NaCl.

Figure 14 shows thrombin cleavage of soluble cell lysate containing GST-SHEL. Increasing amounts of thrombin (indicated in units) were added to soluble cell lysate and analysed by 8% SDS-PAGE. GST can be clearly seen at approximately 26kDa with thrombin concentrations above 0.01U while GST-SHEL decreases. SHEL, at approximately 64 kDa, can be discerned at intermediate thrombin concentrations of 0.05 and 0.1U. Increasing



thrombin further results in removal of the SHEL band and the appearance of three smaller bands (a,b,c) at approximately 45, 34 and 22kDa.

Figure 15 shows the construction scheme for pSHELF $\delta$ 26A. pSHELF and the aberrant pSHELF $\delta$ mod were both digested with SpeI and BssHII. BssHII cuts both plasmids twice and SpeI once resulting in three fragments. The 5424 and 946bp fragments from pSHELF and the small 338bp fragment from pSHELF $\delta$ mod were purified from agarose gels. The 5424bp fragment was CIP treated to reduce recircularisation and the three fragments ligated overnight at 16°C using DNA ligase. The final product pSHELF $\delta$ 26A contained the desired deletion of exon 26A from the SHEL gene with no other mutations.

15

#### **BEST METHOD OF PERFORMING THE INVENTION**

The recombinant and synthetic procedures used are described in standard texts such as Sambrook et al (1989).

20 Purification of the tropoelastin derivatives and expression products of the invention is also performed using standard techniques with the actual sequence of steps in each instance being governed by the environment from which the molecule is to be purified. By way of example, reference is made to the purification scheme disclosed in PCT/AU93/00655.

Formulations in accordance with the invention are formulated in accordance with standard techniques.

30 The amount of tropoelastin derivative or peptidomimetic molecule that may be combined with a carrier or diluent to produce a single dosage form will vary depending on the situation in which the formulation is to be used and the particular mode of administration.

It will be understood also that specific doses for 35 any particular host may be influenced by factors such as

the age, sex, weight and general health of the host as well as the particular characteristics of the modified tropoelastin being used, and how it is administered.

Injectable preparations, for example, sterile  
5 injectable aqueous or oleagenous suspensions may be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic  
10 parenterally acceptable diluent or solvent. Among the acceptable vehicles or solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium.  
15 For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid and organic solvents find use in the preparation of injectables.

Routes of administration, dosages to be administered  
20 as well as frequency of administration are all factors which can be optimised using ordinary skill in the art.

In addition, the derivatives and expression products may be prepared as topical preparations for instance as anti-wrinkle and hand lotions using standard techniques  
25 for the preparation of such formulations.

They also may be prepared in aerosol form for, for instance, administration to a patient's lungs, or in the form of surgical implants, foods or industrial products by standard techniques.

30

#### **EXPERIMENTAL PROCEDURES**

## **EXAMPLE 1**

### **Materials and Methods**

#### *Reagents*

5           Hirudin, PMSF, human thrombin, human plasma  
kallikrein, human plasmin and human leukocyte elastase  
(HLE) were obtained from Sigma. Bovine trypsin and  
Pefabloc SC were from Boehringer-Mannheim and Pefabloc PK  
was from Pentapharm, Switzerland.

10           **SHEL** was obtained by the method described in  
WO94/14958.

**SHEL $\delta$ 26A** can be derived from SHEL by removing the  
synthetic coding sequence corresponding to exon 26A. A  
comparison of the sequence of SHEL with that of SHEL $\delta$ 26A is  
15       provided at Figure 3. Its protein product is apparently  
identical to a naturally made human splice form of  
tropoelastin.

          The Transformer Mutagenesis Kit (Clontech USA) was  
used with pSHELF (described in WO94/14958) in  
20       accordance with the supplied protocol to remove DNA  
corresponding to exon 26A. The sequence of the  
mutagenic primer used (manufactured by Beckman  
Australia) was:

5' CGG GTT TCG GTG CTG TTC CGG GCG CGC TGG 3'  
which flanked either side of exon 26A by 15 bp  
resulting in its precise deletion. A second selection  
primer, which mutates a unique restriction site to  
5 another restriction site is normally used in the  
protocol but was not in this case since deletion of  
exon 26A also resulted in the deletion of a unique  
restriction site, PmlI. This enzyme was therefore  
used to digest the mutation reaction to linearise any  
10 unmutated parental plasmid and consequently to enrich  
for mutant plasmid in accordance with the  
manufacturer's instructions. The reaction mixture was  
used to transform competent BMH17-18 *mutS* *E. coli*  
defective in mismatch repair, by electroporation which  
15 was performed using a Gene Pulser apparatus (BioRad  
USA) according to a protocol supplied by the  
manufacturer. Electrocompetent cells were made  
according to standard protocol supplied by Clontech.  
Competent cells were stored in aliquots at -80°C.  
20 After electroporation cells were grown for one hour at  
37°C at 280rpm in 1ml LB. The entire culture  
transformed culture was grown overnight in 5ml  
LB+ampicillin. Mixed plasmid DNA containing both  
mutated and parental plasmids was isolated from the  
25 culture using the Qiagen Spin Plasmid isolation kit  
and the plasmid DNA was digested with PmlI to  
linearise the parental plasmid. The plasmid DNA now  
enriched for mutated plasmid was used to transform *E.*  
*coli* HMS174 by electroporation as described above and  
30 transformants selected on LB plates containing 75µg/ml  
ampicillin.

Colonies were grown overnight and plasmid mini-preparations performed in which a single colony was inoculated into 3ml LB+ampicillin media in 10ml screw-topped tubes and grown overnight with shaking at 37°C.

5 Plasmids were extracted following the alkaline lysis protocol from Sambrook et al (1989). For HMS174 two extractions with phenol/chloroform/isoamyl alcohol were performed. Constructs were screened using PmlI and those which were insensitive to digestion were

10 further screened by KpnI/PstI double digestion. Candidate clones were sequenced (as described herein) manually using

6F (5' GGG TGT TGG CGT TGC ACC AG 3') and  
7R (5' TGC ACC TAC AAC ACC GCC CG 3') primers to

15 confirm sequence integrity either side of the deleted region.

Automated sequencing was conducted (using either Sequi-Net (Department of Biochemistry Colorado State University USA) or by SUPAMAC (Sydney University and

20 Prince Alfred Hospital Macromolecular Analysis Centre). DNA was applied after purification by either cesium chloride gradient or Qiagen Tip 20 (Qiagen GmbH Germany) and sequenced using the same primers as for manual sequencing.) using primers

25 1R (5' TGC CTT TGC CGG TTT GTA CG 3')  
3F (5' TCC AGG TGG CTA CGG TCT GC 3')  
3R (5' GAG TAC CTA CGC CTG CGA TAC 3')  
5R (5' GGA GTA CCA ACG CCG TAC TT 3')  
6F (5' GGG TGT TGG CGT TGC ACC AG 3')  
30 7R (5' TGC ACC TAC AAC ACC GCC CG 3')  
pETforward (5' GCA CTC ACT ATA GGG AGA CC 3')  
pETreverse (5' GCC AAC TCA GCT TCC TTT CG 3')

was performed to verify the rest of the sequence. A number of undesired mutations were discovered

35 necessitating further manipulation to the DNA. The mutated DNA is named pSHELF $\delta$ mod.

Sequencing confirmed the region immediately surrounding the deletion was correct. PstI and BssHII restriction sites surrounding the correct region of pSHELF $\delta$ mod was used to remove the desired segment and  
5 reinsert it into into the corresponding site of pSHELF. 6.5 $\mu$ g pSHELF and 7.5 $\mu$ g pSHELF $\delta$ mod were digested with BssHII precipitated and digested with PstI. The appropriate three fragments (Figure 15) were gel purified and ligated using 1U DNA ligase  
10 (Boehringer Mannheim Germany) overnight at 16°C. DNA was transformed into *E. coli* XL1-Blue and transformants selected on plates containing 75 $\mu$ g/ml ampicillin.

Plasmids were isolated by mini-preparations and  
15 screened using BglI digestion. A candidate clone was further analysed by restriction enzyme digestion and automated sequencing was then performed using primers 1R, 3F, 5R, 6F, 7R and T7 forward (5' TAA TAC GAC TCA CTA TAG GG 3') to confirm the entire sequence. The  
20 correct sequence was designated pSHELF $\delta$ 26A.

SHEL $\delta$ 26A displays higher protease resistance than SHEL.

#### **Serum Proteolysis of SHEL**

25 Human serum was obtained from fresh intravenous blood, centrifuged at 2000g to remove red blood cells and then allowed to clot before serum was removed. Aliquots (20 $\mu$ l) were stored at -20°C and thawed when needed. 15 $\mu$ g tropoelastin in 50mM sodium phosphate buffer, pH 7.8 was  
30 incubated with 0.5 $\mu$ l serum in a 20 $\mu$ l reaction for between 1 and 18hr at 37°C. Similar experiments were conducted with or without the prior addition of inhibitors. Inhibitors were added at the following concentrations; 0.5 or 1Uhirudin, 0.5 or 5mM Pefabloc SC, 1 or 5mM PMSF, 25mM

EDTA, 50 or 250 $\mu$ M Pefabloc PK. All inhibitors were dissolved in water except PMSF which was dissolved in isopropanol. Reactions were analysed by 8% SDS-PAGE.

5 Serum-digested peptides to be used for sequencing were purified by the addition of 1.5 volumes n-propanol, followed by 2.5 volumes n-butanol and stirred overnight. The organic solvents were removed by rotary evaporation and peptides resuspended in 50mM sodium phosphate buffer, pH 7.8.

10

### **Proteolytic Assays**

A range of enzyme concentrations was originally used to determine the optimal amount for subsequent experiments. Thrombin (0.01-1U), human plasma kallikrein ( $3 \times 10^{-4}$  to  $3 \times 10^{-3}$ U), human plasmin ( $7 \times 10^{-5}$  to  $4 \times 10^{-7}$ U), bovine trypsin ( $5 \times 10^{-4}$  to  $4 \times 10^{-3}$ U), and human leukocyte elastase ( $1.6 \times 10^{-4}$  to  $3.2 \times 10^{-3}$ U) were added to 10 $\mu$ g SHEL or SHEL $\delta$ 26A in 50mM sodium phosphate buffer pH 7.8 in a total volume of 20 $\mu$ l. All reactions were performed at 37°C for one hour. The degradation profile was analysed by 8, 10 or 12% SDS-PAGE.

20

### **N-terminal sequencing**

Gels were poured using fresh acrylamide stocks and half the usual amounts of APS and TEMED. Gels were allowed to set for 16-24hrs. For simple protein profiles, gels were pre-run at room temperature for four hours at 20mA using 150mM Tris HCl pH8.8 buffer with 10 $\mu$ l/L thioglycollic acid in the upper buffer chamber. Samples were loaded and run at 4°C with fresh buffer for approximately three hours. For more complex profiles gels were pre-run at room temperature in Tris-glycine buffer (25mM Tris HCl, 192mM glycine, 0.1% (w/v) SDS, pH approximately 8.3), fresh buffer added and the gel allowed to equilibrate to room temperature before samples were added and run at 20mA with 10 $\mu$ l/L thioglycollic acid added to the upper chamber. Pre-

30

35

stained standards (Kaleidoscope; Biorad, USA) were used to monitor extent of migration.

Gels were blotted onto polyvinylidene difluoride (PVDF) membrane (ProBlott, Applied Biosystems USA) treated according to manufacturer's instructions, overnight at 70mA using 10mM CAPS pH 11.0, 10% methanol, 10 µl/L thioglycollic acid buffer at 4°C with stirring. Blotting was performed using a Hoefer Transblot apparatus and was used according to manufacturer's instructions. The membrane was stained with 0.1% Coomassie blue-R in 50% methanol and destained in 50% methanol, 10% acetic acid. The membrane was washed with water overnight before being air-dried. Bands were excised with a clean scalpel. Samples were blotted onto PVDF as described above. Bands were excised with a clean scalpel and sequenced by Sydney University and Prince Alfred Hospital Macromolecular Analysis Centre (SUPAMAC) using Applied Biosystems hardware and protocols. Alternatively samples were sent to the Biomolecular Resource Facility Australian National University, Canberra, for sequencing.

#### **Peptide Preparation and Use**

S-GAL, N-VVGSPSAQDEASPLS-C, is a peptide representing the elastin binding domain of EBP (Hinek and Rabinovitch 1994). It was synthesised by Chiron Mimotopes (Australia) and purified by RP-HPLC as follows. Concentrated peptide in 50mM ammonium acetate was treated by RP-HPLC initially by perfusion chromatography (POROS, PerSeptive Biosystems USA) using an R2 reverse phase column (4.6 x 100mm) run at 9ml/min along a 0-100% acetonitrile, 0.1% trifluoroacetic acid (TFA) gradient over 7min was used. Alternatively, a Techogel10 C18 column (2.2 x 25cm) was used with a flow rate of 8ml/min. A 0-100% acetonitrile, 0.1% TFA gradient over 55 min was used after a 10min initial wash with 30% acetonitrile/0.1% TFA. The column was equilibrated for 10min between runs due to its large volume. A maximum of



30-50mg peptide was loaded at any one time. For both methods sample detection was at 214 and 280nm simultaneously. Both methods were performed using Pharmacia (Sweden) pumps and detectors. The solution was removed from the collected samples by lyophilisation and purified peptide weighed to determine yield.

A large molar excess of S-GAL in Milli-Q water (10 to 200 fold) was added to 15µg SHEL in 50mM sodium phosphate pH7.8 made up to a total volume of 40µl and preincubated at 37°C for one hour as suggested by Hinek and Rabinovitch (1994) before the selected protease (kallikrein,  $6-15 \times 10^{-4}$ U; thrombin 0.1-0.2U; trypsin  $2 \times 10^{-3}$ U; plasmin,  $1.5-3.7 \times 10^{-5}$ U; human leukocyte elastase,  $1.6 \times 10^{-3}$ U; serum 1µl) was added according to the optimal amounts determined above for 10 to 80 minutes. Various dilutions of serum from ½-1/50 in 50mM sodium phosphate pH7.8 were used and both SHEL and SHELδ26A were used for each experiment.

A peptide representing a region of SHEL cleaved by a selection of serine proteases: N-AAKAQLRAAAGLGA-C (serine protease site peptide, SPS-peptide) was synthesised by Chiron Mimotopes (Australia) to test whether its presence could protect SHEL from degradation by acting as a competitor. Experiments were conducted in parallel with S-GAL using identical procedures (see above). Both SHEL and SHELδ26A were used. Each reaction was analysed by 10% SDS-PAGE. Gels were scanned by densitometry and the volume of full-length SHEL calculated as follows. Scanning densitometry of stained gels was performed using the Molecular Dynamics Personal Densitometer. Images were analysed and quantitated using ImageQuant software (Version 3.2, Molecular Dynamics USA).

### **Proteolysis During Coacervation**

10mg/ml SHEL in 50mM sodium phosphate pH7.8 and 150mM NaCl was allowed to coacervate at 37°C until cloudy before adding human plasma kallikrein ( $6 \times 10^{-4}$ U), thrombin (1U), plasmin ( $1.5 \times 10^{-5}$ U), trypsin ( $2 \times 10^{-3}$ U), HLE ( $1.6 \times 10^{-3}$ U) and serum (0.75 $\mu$ l) for one hour. Control reactions were performed at 16°C for three hours. Extent of proteolysis was monitored by SDS-PAGE.

#### **10 A. Degradation of SHEL by Serum**

Human tropoelastin was degraded by human serum into discrete bands, resistant to further degradation. The same degradation profile was seen by SDS-PAGE with overnight incubation as with incubations left for one hour (Figure 5a). Figure 5b clearly shows the peptide fragments after purification from serum using butanol. The sizes of the major bands are approximately 50, 45, 35, 28, 27, 25, 22 and 18 kDa, visually similar to that obtained by Romero et al (1986) using porcine tropoelastin. The pattern of peptides produced was reproducible over many separate experiments. Similar results were obtained with SHEL $\delta$ 26A (Figure 5b) but the 22 and 18kDa bands were absent and replaced by a 15kDa band.

#### **25 B. Effect of Protease Inhibitors on Serum Degradation**

Figure 6 shows the amount of full-length SHEL after incubation with serum in the presence of and absence of various protease inhibitors. Wide-spectrum serine protease inhibitors were found to inhibit degradation since both Pefabloc SC and PMSF protected tropoelastin from cleavage (Figure 6). In contrast, EDTA which is an inhibitor of metalloproteases, appeared to promote digestion. Protease inhibitors specific for the serine proteases thrombin and kallikrein were also tested. Hirudin, a highly specific inhibitor of thrombin, did not appear to significantly

inhibit degradation whereas Pefabloc PK, specific for kallikrein, inhibited proteolysis (Figure 6).

### C. Degradation of SHEL with specific proteases

#### 5 *Human thrombin*

Thrombin is able to cleave GST-SHEL extensively and in a reproducible manner. Cleavage of GST-SHEL bound to glutathione agarose was performed by washing and resuspending beads in 1x thrombin cleavage buffer (50mM  
10 Tris-HCl pH8.0, 150mM NaCl, 2.5mM CaCl<sub>2</sub>) and adding human thrombin (Sigma) from 0.1 to 1% (w/v) thrombin:fusion protein at 25°C for one hour (Smith and Johnston 1988). Soluble bacterial lysates used as substrate were incubated similarly with 1x thrombin cleavage buffer, added from a  
15 10x stock. GST (26kDa) was evident on beads by SDS-PAGE but SHEL could not be identified in the supernatant in numerous experiments. To determine whether thrombin was degrading SHEL, the entire cell lysate was subject to cleavage with increasing concentrations of thrombin. 0.01U  
20 thrombin was the lower limit for cleavage but 0.05U and greater are more effective (Figure 14). GST was clearly present. However, with 0.01U thrombin a band at approximately 64kDa could be discerned which may represent SHEL although this was not nearly as intense as the GST  
25 band. With higher thrombin concentrations this band disappeared and smaller fragments at 45, 34 and 22kDa were noted indicating that SHEL was indeed being cleaved by thrombin.

When increasing amounts of thrombin were added to pure  
30 SHEL, four major fragments were identified by SDS-PAGE estimated at 45, 34, 22 and 13 kDa (Figure 7a) in addition to faint minor bands. The sizes of the major products were very similar to those seen with thrombin digests of GST-SHEL lysates.

Even with an excess of thrombin added (1U/10µg SHEL) the smaller bands were resistant to further degradation whilst the 45kDa fragment disappeared. The pattern of degradation did not appear to be the same as the serum produced peptides. When the hirudin was added to reaction, degradation was inhibited (not shown) unlike the results seen with serum. The patterns of degradation seen with SHELδ26A was slightly different with the 22kDa fragment reduced in size to about 15 kDa consistent with the fragment not containing 26A (Figure 7).

#### *Human Plasma Kallikrein*

Like thrombin, increasing amounts of human plasma kallikrein added to SHEL resulted in specific and reproducible degradation. Three major fragments were identified by SDS-PAGE (Figure 8a) estimated to be 45, 22 and 18kDa, in addition to faint minor bands. The major bands at 45kDa and 18kDa were resistant to further degradation whilst the 22kDa fragment eventually disappeared. Again, the pattern of degradation was not identical to that seen by serum. Pefabloc PK could inhibit degradation by plasma kallikrein (not shown). The pattern of degradation of SHELδ26A was somewhat different, with the 22 and 18kDa fragments missing and replaced by a 15kDa fragment (Figure 8b), as was seen for serum.

#### *Bovine Trypsin*

Trypsin digestion of SHEL was very extensive, resulting in complete degradation with prolonged treatment. However, with dilute amounts of enzyme ( $4 \times 10^{-3}$ U) major bands could be identified at approximately 50, 45, 40, 38, 34, 31, 22 and 18kDa, giving an overall pattern similar to serum products (Figure 9a). Indeed, at low enzyme concentrations the trypsin digest profile looked virtually identical to the serum digest profile. However,

trypsin digestion was not easily reproducible due to the vigorous action of trypsin on SHEL. Similar results were obtained using SHEL $\delta$ 26A (Figure 9b) except that the sizes of the smaller fragments below 34kDa were all reduced in size by approximately 4kDa and as for kallikrein and serum, the 22 and 18kDa fragments were replaced by a single fragment at 15kDa.

#### *Human Plasmin*

Using plasmin at low concentrations also gave a profile very similar to both serum and trypsin (Figure 10a) while at high concentration extensive degradation occurred. Major bands could be isolated using low concentration plasmin at 55, 45, 40, 34, 28, 22 and 18kDa, similar but not identical to serum digested products. Similar results were obtained using SHEL $\delta$ 26A (Figure 10b) except that smaller fragments below 34kDa were reduced by approximately 4kDa and the 22 and 18kDa fragments were replaced by 17 and 15kDa fragments.

#### *Human Leukocyte Elastase (HLE)*

HLE resulted in extensive degradation if left for extended period. Using  $1.6 \times 10^{-2}$ U numerous fragments were seen with two prominent fragments at 32 and 18kDa (Figure 11a). Fragments were very difficult to isolate, however, and overdigestion occurred easily. SHEL $\delta$ 26A produced a similar profile but with all fragments appearing 4kDa smaller (Figure 11b).

### **D. Mapping of Protease-Susceptible Sites**

The thrombin, kallikrein, plasmin, trypsin and serum-produced peptides indicated in Figures 5 to 11 by an arrow, were N-terminally sequenced and assigned to regions of SHEL. Peptides corresponded either to the N-terminus of SHEL or to cleavage sites C-terminally adjacent to a Lys or

Arg. Sequences of peptides are shown in Table 1 and the positions of the cleavage sites are indicated diagrammatically in Figure 1.

5 The actual sizes, in kDa, of the fragments shown in Table 1 were determined from the amino acid sequence and are shown in brackets. In some cases, this differed from the apparent size as determined by SDS-PAGE. Curiously, one site between residues 515 and 516 (Arg and Ala) was common to thrombin and kallikrein. In addition, this  
10 same site was also cleaved by human serum. This site was identified by sequencing to be located within 26A. The lack of a second kallikrein-produced fragment in SHEL  $\delta$ 26A is therefore consistent with this site being absent from this isoform. The other serum-produced bands, which were  
15 minor in comparison, were unique and appeared to consist of a mixture of peptides making the designation tentative. These peptides were the same size in both SHEL and SHEL $\delta$ 26A (Figure 7b) indicating that they are predominantly N-terminal and that the other peptide fragment is present at  
20 a much lower level. Any significant proteolysis at these other sites in SHEL $\delta$ 26A should result in a 4kDa reduction in peptide size which was not evident. Due to the rampant degradation seen by both trypsin and plasmin, the smaller fragments were unable to be isolated in sufficient quantity  
25 for sequencing. However, the sizes of the fragments indicate that the 22 and 18kDa fragments of trypsin and plasmin are probably the same sequence as for kallikrein and serum. Each of the plasmin-produced bands sequenced were a mixture of the same identified sequences, not seen  
30 with any other protease or serum, and N-terminal sequence also. Since not all the plasmin and trypsin-produced peptides were able to be identified unambiguously, the likely region of cleavage for these enzymes is not shown in Figure 1.

#### **E. Effect of S-Gal and SPS-peptide on Degradation**

The major serine protease site (R/AAAGLG) identified in SHEL as common to thrombin, kallikrein, serum and probably trypsin and plasmin, was produced with some flanking amino acid residues as a 14 amino acid peptide (SPS-peptide). This was added to proteolytic digests of SHEL and SHEL $\delta$ 26A to assess whether this peptide could inhibit degradation by acting as an alternative site for recognition and cleavage by proteases. In addition, S-GAL, a 15 amino acid peptide corresponding to the elastin binding domain of EBP was produced to assess whether its inhibition of porcine pancreatic elastase (Hinek and Rabinovitch 1994) could be extended to other proteases with tropoelastin-degrading ability. Using a 100:1 molar excess of SPS-peptide to SHEL, more full-length SHEL was evident compared with controls using trypsin, plasmin, kallikrein and serum, judged visually by SDS-PAGE and confirmed by scanning densitometry (Figure 12). The effect was most obvious with short incubations (20 minutes) and was seen with both SHEL and SHEL $\delta$ 26A (not shown). SPS-peptide also resulted in more full-length SHEL using thrombin and HLE but to a lesser extent (Figure 12) but longer incubations with thrombin did appear to show some inhibition (Figure 12). Degradation by HLE, however, was consistently inhibited by S-GAL even with longer incubations when inhibition with SPS-peptide was no longer seen, but was not repressed altogether (Figure 12).

#### **F. Effect of Coacervation on Degradation of SHEL**

SHEL, when in the coacervated state at 37°C was significantly protected from degradation by both thrombin and kallikrein (Figure 13a) but not by plasmin. There was also some inhibition of HLE, trypsin and serum (Figure

13a). This inhibition of degradation was not due to the presence of high concentrations of NaCl in the reaction mixture as control reactions using both lesser concentrations of SHEL that did not coacervate at 37°C (not shown) and reactions performed at lower temperatures not conducive to coacervation, did not show any difference in degradation in the presence or absence of NaCl (Figure 13b).

### Discussion

#### 10 Inhibition Study of Serum Degradation of SHEL

Human serum was able to degrade tropoelastin in a specific and reproducible manner into at least five or six major peptide fragments. The SDS-PAGE banding pattern with serum is visually similar to that of Romero et al (1986).  
15 Various inhibitor studies confirmed the protease to be a serine protease which could be inhibited by the broad spectrum serine protease inhibitors Pefabloc SC and PMSF. Metalloprotease activity was not a major contributor as evidenced by the lack of inhibition by EDTA. Indeed, EDTA  
20 appeared to enhance degradation by serum perhaps by modulating the action of an inhibitor of serum proteases. It is expected that metalloproteases are a major source of proteolytic activity when tropoelastin is exposed to wound exudate. Thrombin did not appear to be responsible for the  
25 majority of serum cleavage because the degradation by serum was not substantially inhibited by the thrombin-specific inhibitor hirudin, yet controls using tropoelastin and thrombin were inhibited. Pefabloc PK, specific for kallikrein inhibited degradation. Romero et al (1986)  
30 found that incubation of tropoelastin with kallikrein resulted in a somewhat similar profile to its incubation with serum. The present inhibitor studies with PefablocPK are therefore consistent with kallikrein and/or proteases with similar behaviour being involved. The inhibitor



Pefabloc PK is, however, not completely specific for kallikrein. According to data supplied by the manufacturer, the inhibitor constant for plasma kallikrein is  $0.7\mu\text{mol/L}$  while the next most likely enzyme to be inhibited after kallikrein is trypsin with an inhibitor constant of  $1.3\mu\text{mol/L}$  followed by plasmin at  $10\mu\text{mol/L}$ . Thus, if present in excess Pefabloc PK may be inhibiting these enzymes also. However, the lowest concentration at which complete inhibition was seen ( $50\mu\text{M}$ ) was the manufacturer's recommended amount for inhibition of kallikrein in plasma samples.

#### Identification of Serum Proteolysis

A number of enzymes have been proposed to be responsible for the serum degradation of tropoelastin. Kallikrein (Romero et al 1986) and plasmin (McGowan et al 1996) have both been put forward as potential sources of proteolysis while a trypsin-like protease was thought to be responsible for the degradation products seen when tropoelastin was isolated from tissues (Mecham and Foster 1977). A visual comparison of SHEL degradation products from serum with the individual protease digestion products revealed only a limited similarity with thrombin and kallikrein-produced peptides while trypsin and plasmin digests appeared more similar to serum-digested peptides but only when used at low concentration. Higher concentrations and/or longer incubations completely degraded SHEL and SHEL $\delta$ 26A in contrast to long incubations with serum which did not change the pattern greatly.

Increasing amounts of thrombin easily degraded SHEL but only three major fragments were noted, unlike serum-produced peptides where 5-6 fragments were noted. Coupled with the observation from the inhibitor studies that the thrombin-specific inhibitor hirudin did not substantially

reduce serum degradation, thrombin does not appear to be the major enzyme involved in serum proteolysis of SHEL. This was corroborated by sequencing of the peptide products which showed that although one of the two sites recognised by thrombin was likewise recognised by serum, the other site was not. This may have been a consequence of low thrombin concentration but this is unlikely since both sites are recognised to a similar extent (Figure 9).

Similarly, the profile of SHEL seen after kallikrein digestion only showed limited similarity to the serum produced profile i.e. the presence of a 45kDa fragment and two fragments around 20kDa. Sequencing of the peptides showed that both the sites recognised by kallikrein were recognised by serum. The other serum-produced fragments, however, were not seen as major products of kallikrein digestion although some other fragments were present at a very low level (Figure 8). Long incubations with kallikrein (overnight) failed to increase the intensity of other fragments nor increase to resemble serum digestion products (not shown), indicating that kallikrein was unlikely to be responsible for the additional serum-produced fragments. The sequencing data, effect of a kallikrein specific protease inhibitor and visual appearance of the digestion products by SDS-PAGE are all consistent with the involvement of kallikrein in serum digestion. However the presence of other serum peptide fragments not seen as major products of kallikrein digestion indicates that kallikrein alone is not responsible for the pattern seen in serum digests.

In contrast to thrombin and kallikrein, treatment with plasmin and trypsin resulted in extensive degradation which could completely degrade SHEL if incubated for extended periods. The degradation profile seen with plasmin was quite unlike that seen by McGowan et al (1996) where only 68 and 45kDa bands were seen suggesting that the

degradation had not proceeded very far in that case. Each of these digestion profiles were more similar to serum products than either thrombin or kallikrein. By visual inspection trypsin and plasmin appeared almost identical to  
5 serum digests and each other but only at a low concentration.

There was some difficulty in the sequencing of plasmin and trypsin peptides. The plasmin-produced peptides that were sequenced were found to consist of a  
10 mixture of at least two overlapping sequences at 78/79 and 81/82 (K/AAK and K/AGA) which were the same in all of the peptide fragments sequenced. In addition, sequence from the N-terminus of SHEL was also present, which made these peptides very difficult to identify unambiguously. The  
15 presence of the same peptides throughout each fragment may be an artifact resulting from this sequence co-migrating through the entire gel with other peptides and so contaminating each subsequent peptide (J. McGovern Biomolecular Resource Facility, John Curtin School of  
20 Medical Research, Australian National University, personal communication). This may have been compounded by the low levels of peptide obtained for each fragment due to the rampant degradation by plasmin.

Similarly, low levels and poor resolution made it  
25 difficult to obtain sequence for the smaller trypsin peptides. However, clear sequence data were obtained for the larger fragments which all corresponded to N-terminal sequences as was the case for the same peptides from serum. This coupled with the observation that Pefabloc PK could  
30 also inhibit trypsin in controlled reactions (not shown) and the visual similarity of peptide fragments is consistent with trypsin-like enzyme involvement with serum proteolysis but the lack of sequence data for the more informative smaller fragments means that the identification  
35 is not definitive. Similarly, the visual similarity is

also consistent with plasmin involvement but this was not  
able to be confirmed by sequencing. Since serum proteolysis  
was more defined and limited than either plasmin or trypsin  
alone, this indicates that the presence of trypsin-like  
5 activity is probably much lower in serum and/or is more  
easily destroyed.

HLE digestion profile was also extensive but was  
different to serum, trypsin and plasmin. HLE is a serine  
elastase and cleaves predominantly at Val residues (Keil  
10 1992). The difference between elastase digests of SHEL and  
SHEL $\delta$ 26A was more notable as most fragments, including the  
largest ones, were smaller in SHEL $\delta$ 26A, indicating that  
digestion was occurring preferentially from the N-terminal  
end which does not appear to be the case for the other  
15 enzymes or serum. HLE involvement in serum proteolysis is  
therefore unlikely.

In summary, by N-terminal sequencing, visual  
inspection of the degradation profiles by comparison with  
that of serum and the effect of the inhibitors the results  
20 are consistent with involvement of kallikrein and/or  
protease(s) capable of giving a comparable cleavage  
pattern, in addition to at least one other enzyme probably  
present at a lower level. Plasmin or another trypsin-like  
enzyme or combination of enzymes are the most likely to be  
25 involved in the serum digestion of SHEL. Detectable  
thrombin and HLE activity in serum are unlikely.

#### Mapping of Protease Sensitive Sites

The pattern of degradation of purified tropoelastin  
30 seen by others is similar to the sizes of peptides  
generated by our proteolysis experiments. The sizes seen  
by Mecham and Foster (1977) by their trypsin-like protease

associated with tropoelastin, 57,45, 36, 24.5 and 13-14kDa are very similar to the number and sizes of peptides generated by serum and the individual serine proteases on both SHEL and SHEL $\delta$ 26A indicating that cleavage may be occurring in the same or similar places. A similar profile was seen with tropoelastin from human fibroblast cell culture (Davidson and Sephel 1987). Sequencing confirmed that one site between residues 515 and 516 was common to thrombin, kallikrein and serum and from the SDS-PAGE pattern, probably also plasmin and trypsin. All the peptides sequenced confirmed that cleavage occurred after a Lys or Arg as expected for many serine proteases (Keil 1992). However, tropoelastin contains a large number of Lys and Arg (35 and 10 respectively) yet only a small number of these residues were actually recognised and cleaved. The fact that these same sites may be recognised by different serine proteases may be due to their accessibility and/or the surrounding amino acids.

Preferred recognition sites for kallikrein and thrombin are strongly influenced by the adjacent amino acid residues (Chang 1985; Keil 1992) but it would not have been possible a priori to predict where preferential cleavage occurs in human tropoelastin. For example, kallikrein cleaves preferentially at Arg residues preceded by a bulky residue (Keil 1992). Both sites identified by N-terminal sequencing fall into this category, with Leu-Arg at 515 and Arg-Arg at 564. However, for example, another Arg preceded by a Leu at 571 does not appear to be recognised. The highly specific and limited proteolysis of SHEL and SHEL $\delta$ 26A by kallikrein has allowed kallikrein treatment to be used to produce isolated C-terminal portions of tropoelastin for further study (S. Jensen and A.S. Weiss unpublished). The thrombin sites identified, however, do not fit the preferred sites for thrombin. Thrombin recognises predominantly P2-Lys/Arg-P1' where either P2 or

P1' are Gly or P4-P3-Pro-Arg/Lys-P1'-P2", where P4 and P3 are hydrophobic and P1' and P2' are non-acidic residues (Chang 1985) with Arg greatly favoured over Lys (Keil 1992). Neither SHEL nor SHEL $\delta$ 26A contain these exact sites  
5 although the site at 152 (Lys-Pro-Lys-Ala-Pro) is similar to the latter recognition site of P3-Pro-Lys-P1'-P2'. Which sites are recognised and cleaved may therefore be under the influence of tropoelastin secondary structure. Trypsin cleaves predominantly at Arg and Lys with a  
10 preference for Arg, while plasmin preferentially cleaves at Lys (Keil 1992). Since there are more Lys than Arg in tropoelastin, it would be expected that these proteases would cleave more extensively as is shown to be the case.

#### Protection from Degradation

15 Experiments have demonstrated that EBP can protect tropoelastin from degradation by binding primarily to the VGVAPG sequence of tropoelastin (Mecham et al 1989). A peptide S-GAL which represents the elastin binding site of EBP has been used previously to model the interaction  
20 (Hinek and Rabinovitch 1994). It has been noted that S-GAL and EBP have some homology with the N-terminal sequence of proteases such as kallikrein, HLE and plasmin and are therefore proposed to bind to the same sequence in tropoelastin, thus acting as competitive inhibitors of the  
25 proteases (Hinek and Rabinovitch 1994; Hinek et al 1993). Hinek and Rabinovitch (1994) showed that S-GAL could significantly inhibit degradation of elastin by porcine pancreatic elastase and inferred that HLE and other serine proteases could be similarly inhibited from degrading  
30 tropoelastin. In this work, the use of S-GAL did not show any significant or consistent inhibition of proteolysis of SHEL or SHEL $\delta$ 26A by serum, trypsin, plasmin or kallikrein although some inhibition could be seen with thrombin. However, significant and reproducible inhibition was seen

with HLE but complete inhibition of degradation could not be achieved, even with the large excess of S-GAL used. The S-GAL used was HPLC-purified to remove any truncated products and it may be possible that the peptide was  
5 damaged or irreversibly denatured by this process. However, samples of S-GAL which were not HPLC purified gave similar results (not shown). The mass spectroscopy data supplied by the manufacturer indicated that the correct product was synthesised. Therefore S-GAL either did not  
10 bind to SHEL or SHEL $\delta$ 26A very effectively or was easily displaced by the protease. Alternatively, the proteases may be binding to more than one site on tropoelastin and are therefore not effected by S-GAL.

In summary, S-GAL showed partial inhibition of  
15 tropoelastin degradation by HLE and thrombin but inhibition was not as thorough as seen by Hinek and Rabinovitch (1994) using porcine pancreatic elastase. More extensive inhibition of other proteases and serum could not be shown consistently. N-terminal sequencing data revealed  
20 one site in SHEL which was commonly recognised by thrombin, kallikrein, serum and probably trypsin and plasmin. This site and its flanking amino acids was synthesised and this SPS-peptide added to the proteolytic digests of SHEL and SHEL $\delta$ 26A. This peptide was not expected to bind to  
25 tropoelastin but simply act as a competitor by being recognised by the protease thus slowing degradation of SHEL and SHEL $\delta$ 26A. There was reproducible evidence of protection from degradation of SHEL and SHEL $\delta$ 26A by the presence of SPS-peptide. The amount of full-length protein  
30 was greater in the presence of SPS-peptide than in the presence of S-GAL or control digestions and was similar for both isoforms. This was most notable in the presence of low enzyme concentrations or shorter incubations and was most obvious with trypsin, plasmin, kallikrein and serum

although protection from the other proteases was noted  
although at a reduced level. This indicates that each of  
the proteases and serum could recognise this peptide to  
some extent and therefore this is a potential inhibitor of  
5 proteolysis of tropoelastin.

There is no direct evidence that SPS-peptide is  
cleaved by any protease. However, the presence of a  
similar amount of a different peptide (S-GAL) did not exert  
the same effect. Thus the effect of SPS-peptide is  
10 probably not simply due to the non-specific presence of a  
peptide in the reaction. SPS-peptide is therefore likely  
to be interacting directly with the proteases (or  
tropoelastin) to exert its effect. SPS-peptide may allow  
full-length tropoelastin to persist longer in the presence  
15 of proteases, including human serum.

In summary, the inhibition of degradation of SHEL and  
SHEL $\delta$ 26A by S-GAL was only noted significantly with HLE but  
more extensive protection could not be shown. However a  
reproducible inhibition was seen in the presence of SPS-  
20 peptide with each protease and serum, and was most notable  
with trypsin, kallikrein and serum. This peptide provides  
an alternative site for interaction with proteases and  
results in the persistence of full-length tropoelastin for  
longer periods.

25

#### Proteolysis of Coacervated Tropoelastin

Coacervation of SHEL and SHEL $\delta$ 26A at 37°C resulted in  
significant protection from proteolysis by kallikrein and  
thrombin and to a lesser extent by HLE, trypsin and serum.  
30 No protection was seen from attack by plasmin. The  
presence of 150mM NaCl did not appear to cause the  
inhibition since the same reactions performed under



conditions not conducive to coacervation (16°C) were digested to a similar extent in the presence or absence of NaCl. Although it is possible that a simple change in conformation at 37°C could result in altered proteolytic susceptibility, this is unlikely since coacervated and non-coacervated SHEL both at 37°C were digested at different rates. The inhibition of proteolysis is therefore probably due to steric restriction in the coacervate. Of the enzymes tested, the activity of kallikrein was most significantly inhibited by coacervation. From the N-terminal sequencing results, kallikrein predominantly recognises only two sites in SHEL, both of which are in close proximity, and only one in SHEL826A. The coacervation of tropoelastin appears to mask these sites making them less accessible to kallikrein. With thrombin, the inhibition was not as complete as with kallikrein. Thrombin recognises predominantly two sites in SHEL also but these are more distant from each other. The process of coacervation may mask these sites but if either site is slightly more accessible proteolysis would result and consequently allow easier access to the second site. Other proteases (HLE, trypsin, plasmin) and also serum recognise and cleave at many more sites within SHEL making efficient masking of all sites by coacervation unlikely and resulting in some sites remaining available for recognition and proteolysis to occur. Thus, these proteases are not as significantly inhibited by coacervation. These results indicate that in the extracellular matrix, coacervation of tropoelastin may serve an additional role to those already proposed by providing to a certain extent, protection from proteolysis including that caused by human serum. These results could be extended to the nascent elastic fibre where newly laid tropoelastin in the coacervate would be largely protected from extracellular proteases before cross-linking makes this protection essentially permanent.

Possible consequences of serum degradation of tropoelastin

It is clear from these results and those of others that serum contains factors capable of degrading tropoelastin. A number of serine proteases present in human blood have been shown here to be able to degrade tropoelastin specifically and reproducibly. Thus tropoelastin when secreted by cells into the extracellular matrix is vulnerable to extensive degradation prior to being insolubilised by lysyl oxidase and cross-linked. This is especially significant in blood vessels where damaged vessels may contain a number of these proteases during normal blood coagulation. Any tropoelastin secreted at this time and not protected, for example by EBP or by coacervation, would be fragmented. These results suggest that coacervation may indeed provide some protection from digestion as seen with the inhibition of degradation of coacervated SHEL (Figure 13). However, protection is by no means complete. It has previously been suggested that tropoelastin may be under negative feedback autoregulation and upon accumulation in the extracellular matrix may inhibit the production of elastin mRNA (Foster and Curtiss 1990). Elastin peptides produced by proteases such as elastase have been shown to produce negative feedback inhibition when added to undamaged fibroblast cultures while stimulating tropoelastin production in protease damaged cultures (Foster et al 1990). It has been suggested that serine protease mediated proteolysis of tropoelastin may be an important modulator of tropoelastin production and that plasmin may be involved in this process (McGowan et al 1996). Our results are consistent with this proposal although the specific enzyme(s) proposed differ slightly.

It is interesting to note that most of the cleavages identified in serum occur in the C-terminal half of the tropoelastin molecule and that most of the larger fragments

were from the N-terminus (Figure 1, Table 1). Thus the action of proteases in serum on tropoelastin serves to degrade the C-terminal portion leaving a large N-terminal segment. These shortened molecules may not be incorporated  
5 into newly synthesised or growing elastic fibers due to the absence of the highly conserved C-terminus which is shown to be responsible for binding with microfibrillar proteins (Brown-Ausburger et al 1996; 1994). This is analogous to the case in supra-  
10 valvular aortic stenosis, where an elastin gene truncation results in tropoelastin missing the C-terminus with the result of severe aortic disease (Ewart et al 1994). Similarly, in fetal lamb ductus arteriosus a truncated tropoelastin missing the C terminus is not  
15 incorporated into the elastic fibre (Hinek and Rabinovitch 1993). The action of serum on human tropoelastin therefore results in tropoelastin molecules which may not be rendered insoluble and may persist in the extracellular matrix. Any fibers cross-linked may be aberrant due to improper alignment, resulting in a loss of elastic properties and  
20 strength. The persistence of soluble peptides may serve to inhibit further tropoelastin production by negative feedback inhibition (Foster and Curtiss 1990). At the same time peptides are chemotactic, as demonstrated by several studies (Bisaccia et al 1994; Grosso and Scott 1993) and  
25 may serve to recruit tissue repairing cells to the site of injury, accelerating repair of the wound. Chemotactic peptides may differ in efficacy from for example SHEL and SHEL $\delta$ 26A.

### Conclusion

30 Human serum was shown to be capable of degrading SHEL and SHEL $\delta$ 26A into a number of discrete fragments. This activity was confirmed to be from a serine protease and the regions of susceptibility to serum were precisely mapped by N-terminal sequencing. A number of other serine proteases

were shown to be capable of degrading SHEL and SHEL $\delta$ 26A. From the pattern of degradation, use of selective inhibitors and N-terminal sequencing the protease responsible for serum degradation was consistent with a  
5 trypsin-like protease but kallikrein or kallikrein -like behaviour is also a likely contributor. Significant or consistent inhibition of proteolysis did not take place using S-GAL except with thrombin and HLE but reproducible  
10 inhibition was provided by SPS-peptide. However, the process of coacervation was shown to provide the most significant protection against proteolysis including by serum and was most notable for proteases which cleaved a limited number of sites.

Cleavage of SHEL and SHEL $\delta$ 26A with  
15 metalloproteinases to generate reproducible patterns with apparently preferred cleavage sites has also been demonstrated.

#### **INDUSTRIAL APPLICATION**

20 The derivatives and expression products of the invention are of use in *inter alia* the medical, pharmaceutical, veterinary and cosmetic fields as tissue bulking agents, and agents for cellular chemotaxis, proliferation and growth inhibition, in particular of  
25 smooth muscle cells, epithelial cells, endothelial cells, fibroblasts, osteocytes, chondrocytes and platelets.

**TABLE 1: N-terminal Sequences of Protease-Produced Tropoelastin Peptides**

	Size (kDa) *	Sequence†	Position
thrombin	45	GGVPGAIPG	
	34	<b>K</b> /APGVGGAF	152/153
	22 (19)	<b>R</b> /AAAGLG	515/516
kallikrein	45	GGVPGAIPG	
	22 (19)	<b>R</b> /AAAGLG	515/516
	18 (15)	<b>R</b> /SLSPELREGD	564/565
trypsin	55	GGVPGAIPG	
	45	GGVPGAIPG	
	40	GGVPGAIPG	
	34	GGVPGAIPG	
plasmin	55	GGVPGAIP	
	45	<b>K</b> /AAKAGAGL + GGVPGAIP	78/79
	40	<b>K</b> /AAKAGAGL + <b>K</b> /AGAGLGGV	78/79 + 81/82
	34	<b>K</b> /AAKAGAGL + <b>K</b> /AGAGLGGV	78/79 + 81/82
	28	<b>K</b> /AAKAGAGL + <b>K</b> /AGAGLGGV	78/79 + 81/82
serum	50	GGVPGAIPGGVP	
	45	GGVPGAIPGG	
	34	GGVPGAIPGGVP	
	28 (25)	GGVPGAIPG + <b>K</b> /AAQFGLVPGV(?)‡	441/442
	27	GGVPGAIPGGVPGGFYPG	
	25 (20)	GGVPGAIPG + <b>K</b> /SAAKVAKAQ(?)	503/504
	22 (19)	<b>R</b> /AAAGLG	515/516
	18 (15)	<b>R</b> /SLSPELRE	564/565
	13	GGVPGAIP	
<p>* Size of fragments are calculated from SDS-PAGE and are approximate. Sizes in brackets are the sizes determined from the position of the cleavage determined by N-terminal sequencing.</p> <p>† A slash (/) indicates an internal cleavage site adjacent to an R or K residue (bold). N-terminal sequence of residues to the right of these sites was obtained allowing the precise location of the cleavage site to be allocated and the exact size of the fragment to be calculated.</p> <p>‡ A question mark (?) indicates that this designation is tentative. The peptide is likely to be present at a very low level and as a mixture with other peptides.</p>			

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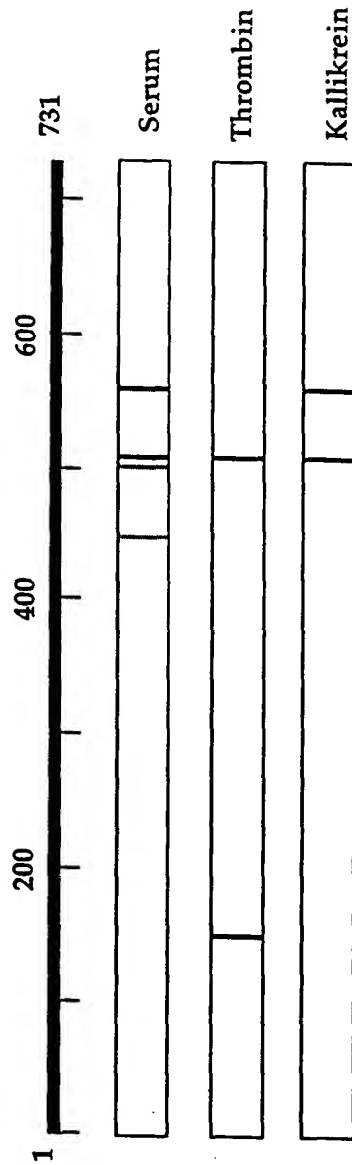


Fig. 1

Schematic diagram showing the relative positions of protease sites identified by N-terminal sequencing for serum, kallikrein and thrombin. Major sites are indicated with a solid bar while minor sites are indicated with a stippled bar. Since plasmin fragments all contained the same N-terminal sequence the site of cleavage could not be identified unambiguously. The trypsin fragments identified similarly all contained the same N-terminal sequence. Therefore, the likely regions of cleavage for plasmin and trypsin are not shown.

1 GATCCATGGGTGGCGTTCCGGGTGCTATCCCGGGTGGCGTTCCGGGTGGTGTATTCTACC 60  
 GTACCCACCGCAAGGCCCACGATAGGGCCCACCGCAAGGCCCACCACATAAGATGG  
 S M G G V P G A I P G G V P G G V F Y P  
 ↑  
 start of mature processed protein

61 CAGGCGCGGGTCTGGGTGCACTGGGCGGTGGTGCCTGGGCCCCGGGTGGTAAACCGCTGA 120  
 GTCCGCGCCCAGACCCACGTGACCCGCCACCACGCGACCCGGGCCCACCATTGCGCGACT  
 G A G L G A L G G G A L G P G G K P L K

121 AACCGGTTCCAGGCGGTCTGGCAGGTGCTGGTCTGGGTGCAGGTCTGGGCGCGTTCCCGG 180  
 TTGGCCAAGGTCCGCCAGACCGTCCACGACCAGACCCACGTCCAGACCCGCGCAAGGGCC  
 P V P G G L A G A G L G A G L G A F P A

181 CGGTTACCTTCCCGGGTGTCTGGTTCCGGGTGGCGTTGCAGACGCAGCTGCTGCGTACA 240  
 GCCAATGGAAGGGCCCACGAGACCAAGGGCCCACCGCAACGTCTGCGTCGACGACGCATGT  
 V T F P G A L V P G G V A D A A A A Y K

241 AAGCGGCAAAGGCAGGTGCGGGTCTGGGCGGGGTACCAGGTGTTGGCGGTCTGGGTGTAT 300  
 TTCGCCGTTTCCGTCCACGCCAGACCCGCCCCATGGTCCACAACCGCCAGACCCACATA  
 A A K A G A G L G G V P G V G G L G V S

301 CTGCTGGCGCAGTTGTTCCGCAGCCGGGTGCAGGTGTAAAACCGGGCAAAGTTCCAGGTG 360  
 GACGACCGCGTCAACAAGGCGTCGGCCCCACGTCCACATTTGGCCCCGTTTCAAGGTCCAC  
 A G A V V P Q P G A G V K P G K V P G V

361 TTGGTCTGCCGGGCGTATACCCGGGTGGTGTCTGCCGGGCGCGCGTTTCCCAGGTGTTG 420  
 AACCAGACGGCCCCGCATATGGGCCCCACCACAAGACGGCCCCGCGCGCAAAGGGTCCACAAC  
 G L P G V Y P G G V L P G A R F P G V G

Fig. 2(1)

421	GTGTACTGCCGGGCGTTCCGACCGGTGCAGGTGTTAAACCGAAGGCACCAGGTGTAGGCG CACATGACGGCCCGCAAGGCTGGCCACGTCCACAATTTGGCTTCCGTGGTCCACATCCGC	480
	V L P G V P T G A G V K P <u>K A P G V G G</u>	
481	GCGCGTTTCGCGGGTATCCCGGGTGTGGCCCGTTTCGGTGGTCCGCAGCCAGGCGTTCCGC CGCGCAAGCGCCCATAGGGCCCAACCGGGCAAGCCACCAGGCGTCGGTCCGCAAGGCG	540
	<u>A F</u> A G I P G V G P F G G P Q P G V P L	
541	TGGGTTACCCGATCAAAGCGCCGAAGCTTCCAGGTGGCTACGGTCTGCCGTACACCACCG ACCCAATGGGCTAGTTTCGCGGCTTCGAAGGTCCACCGATGCCAGACGGCATGTGGTGGC	600
	G Y P I K A P K L P G G Y G L P Y T T G	
601	GTAAACTGCCGTACGGCTACGGTCCGGGTGGCGTAGCAGGTGCTGCGGGTAAAGCAGGCT CATTTGACGGCATGCCGATGCCAGGCCACCGCATCGTCCACGACGCCCATTTTCGTCCGA	660
	K L P Y G Y G P G G V A G A A G K A G Y	
661	ACCCAACCGGTACTGGTGTGGTCCGCAGGCTGCTGCGGCAGCTGCGGGCAAGGCAGCAG TGGGTTGGCCATGACCACAACCGAGCGTCCGACGACGCCGTCGACGCCGCTTCCGTGCTC	720
	P T G T G V G P Q A A A A A A A K A A A	
721	CAAAATTCGGCGCGGGTGCAGCGGGTGTTCGCGGGCGTAGGTGGTGCTGGCGTTCCGG GTTTAAAGCCGCGCCACGTGCGCCACAAGACGGCCCCCATCCACCACGACCGCAAGGCC	780
	K F G A G A A G V L P G V G G A G V P G	
781	GTGTTCCAGGTGCGATCCCGGGCATCGGTGGTATCGCAGGCGTAGGTACTCCGGCGGCGC CACAAGGTCCACGCTAGGGCCCGTAGCCACCATAGCGTCCGCATCCATGAGGCCGCGCGC	840
	V P G A I P G I G G I A G V G T P A A A	
841	CTGCGGCTGCGGCAGCTGCGGCGAAAGCAGCTAAATACGGTGCGGCAGCAGGCCTGGTTC GACGCCGACGCCGTCGACGCCGCTTTCGTGATTATGCCACGCCGTCGTCCGGACCAAG	900
	A A A A A A A K A A K Y G A A A G L V P	

Fig 2(2)

901	CGGGTGGTCCAGGCTTCGGTCCGGGTGTTGTAGGCGTTCCGGGTGCTGGTGTTCGGGGCG GCCCACCAGGTCCGAAGCCAGGCCCAACATCCGCAAGGCQCACGACCACAAGCCCCGC	960
	G G P G F G P G V V G V P G A G V P G V	
961	TAGGTGTTCCAGGTGCGGGCATCCCGGTTGTACCGGGTGCAGGTATCCCGGGCGCTGCGG ATCCACAAGGTCCACGCCCCTAGGGCCAACATGGCCACGTCCATAGGGCCC GCGACGCC	1020
	G V P G A G I P V V P G A G I P G A A V	
1021	TTCCAGGTGTTGTATCCCCGGAAGCGGCAGCTAAGGCTGCTGCGAAAGCTGCGAAATACG AAGGTCCACAACATAGGGGCCTTCGCCGTCGATTCCGACGACGCTTTCGACGCTTTATGC	1080
	P G V V S P E A A A K A A A K A A K Y G	
1081	GAGCTCGTCCGGGCGTTGGTGTGGTGGCATCCCGACCTACGGTGTAGGTGCAGGCGGTT CTCGAGCAGGCCCGCAACCACAACCACCGTAGGGCTGGATGCCACATCCACGTCCGCCAA	1140
	A R P G V G V G G I P T Y G V G A G G F	
1141	TCCCAGGTTTCGGGCGTTGGTGTGGTGGCATCCCGGGTGTAGCTGGTGTTCGGTCTGTTG AGGGTCCAAAGCCGCAACCACAACCACCGTAGGGCCCACATCGACCACAAGGCAGACAAC	1200
	P G F G V G V G G I P G V A G V P S V G	
1201	GTGGCGTACCGGGTGTGGTGGCGTTCCAGGTGTAGGTATCTCCCCGGAAGCGCAGGCAG CACCGCATGGCCCCACAACCACCGCAAGGTCCACATCCATAGAGGGGCCTTCGCGTCCGTC	1260
	G V P G V G G V P G V G I S P E A Q A A	
1261	CTGCGGCAGCTAAAGCAGCGAAGTACGGCGTTGGTACTCCGGCGGCAGCAGCTGCTAAAG GACGCCGTCGATTTCGTCGCTTCATGCCGCAACCATGAGGCCGCGTCGTCGACGATTTC	1320
	A A A K A A K Y G V G T P A A A A A K A	
1321	CAGCGGCTAAAGCAGCGCAGTTCGGACTAGTTCGGGGCGTAGGTGTTGCGCCAGGTGTTG GTCGCCGATTTCGTCGCGTCAAGCCTGATCAAGGCCCGCATCCACAACGCGGTCCACAAC	1380
	A A <u>K A A Q F G L V P G V</u> G V A P G V G	

FIG. 2(3)

1381 GCGTAGCACCGGGTGTGGTGTGCTCCGGGCGTAGGTCTGGCACCGGGTGTGGCGTTG 1440  
CGCATCGTGGCCCACAACCACAACGAGGCCCGCATCCAGACCGTGGCCCACAACCGBAAC  
V A P G V G V A P G V G L A P G V G V A

1441 CACCAGGTGTAGGTGTGGCGCGGGCGTTGGTGTAGCACCGGGTATCGGTCCGGGTGGCG 1500  
GTGGTCCACATCCACAACGCGGCCCGCAACCACATCGTGGCCCATAGCCAGGCCACCBC  
P G V G V A P G V G V A P G I G P G G V

1501 TTGCGGCTGCTGCGAAATCTGCTGCGAAGGTTGCTGCGAAAGCGCAGCTGCGTGCAGCAG 1560  
AACGCCGACGACGCTTTAGACGACGCTTCCAACGACGCTTTCGCGTCGACGCACGTCTGTC  
A A A A K S A A K V A A K A Q L R A A A

---

1561 CTGGTCTGGGTGCGGGCATCCCAGGTCTGGGTGTAGGTGTGGTGTTCGGGCCTGGGTG 1620  
GACCAGACCCACGCCCCGTAGGGTCCAGACCCACATCCACAACCACAAGGCCCGGACCCAC  
G L G A G I P G L G V G V G V P G L G V

---

1621 TAGGTGCAGGGGTACCGGGCCTGGGTGTGGTGCAGGCGTTCCGGGTTTCGGTGTGGCG 1680  
ATCCACGTCCCCATGGCCCGGACCCACAACCACGTCCGCAAGGCCCAAAGCCACGACCCG  
G A G V P G L G V G A G V P G F G A G A

1681 CGGACGAAGGTGTACGTCTCCCTGTCTCCAGAACTGCGTGAAGGTGACCCGTCTCTT 1740  
GCCTGCTTCCACATGCAGCAAGGGACAGAGGTCTTGAAGCACTTCCACTGGGCAGGAGAA  
D E G V R R S L S P E L R E G D P S S S

---

1741 CCCAGCACCTGCGGTCTACCCCGTCTCTCCACGTGTTCCGGGCGCGCTGGCTGCTGCGA 1800  
GGGTGCTGGACGGCAGATGGGGCAGGAGAGGTGCACAAGGCCCGCGCGACCGACGACGCT  
Q H L P S T P S S P R V P G A L A A A K

1801 AAGCGGCGAAATACGGTGCAGCGGTTCCGGGTGTACTGGGCGGTCTGGGTGCTCTGGGCG 1860  
TTCGCGCTTTATGCCACGTGCGCAAGGCCACATGACCCGCCAGACCCACGAGACCCGC  
A A K Y G A A V P G V L G G L G A L G G

Fig. 2(4)

1861	GTGTTGGTATCCCGGGCGGTGTTGTAGGTGCAGGCCAGCTGCAGCTGCTGCTGCGGCAA CACAACCATAGGGCCCGCCACAACATCCACGTCCGGGTCGACGTGACGACGACGCCGTT	1920
	V G I P G G V V G A G P A A A A A A A K	
1921	AGGCAGCGGCGAAAGCAGCTCAGTTCGGTCTGGTTGGTGCAGCAGGTCTGGGCGGTCTGG TCCGTCGCCGCTTTTCGTCGAGTCAAGCCAGACCAACCACGTGTCAGACCCGCCAGACC	1980
	A A A K A A Q F G L V G A A G L G G L G	
1981	GTGTTGGCGGTCTGGGTGTACCGGGCGTTGGTGGTCTGGGTGGCATCCCGCCGGCGGGCGG CACAACCGCCAGACCCACATGGCCCGCAACCACCAGACCCACCGTAGGGCGGCCGCCGCC	2040
	V G G L G V P G V G G L G G I P P A A A	
2041	CAGCTAAAGCGGCTAAATACGGTGCAGCAGGTCTGGGTGGCGTTCTGGGTGGTGTGGTC GTCGATTTTCGCCGATTTATGCCACGTGTCAGACCCACCGCAAGACCCACCACGACCAG	2100
	A K A A K Y G A A G L G G V L G G A G Q	
2101	AGTTCCCACTGGGCGGTGTAGCGGCACGTCCGGGTTTCGGTCTGTCCCGATCTTCCCAG TCAAGGGTGACCCGCCACATCGCCGTGCAGGCCCAAAGCCAGACAGGGGCTAGAAGGGTC	2160
	F P L G G V A A R P G F G L S P I F P G	
2161	GCGGTGCATGCCTGGGTAAAGCTTGCGGCCGTAAACGTAAATAATGATAG CGCCACGTACGGACCCATTTTGAACGCCGGCATTTGCATTTATTACTATCCTAG	2210
	G A C L G K A C G R K R K * * *	

Fig. 2(5)

SHEL X SHELdelta26A

```

1  GGVPGAIPGGVPGGVFFPGAGLGGALGGGALGPGGKPLKPVPGGLAGAGLG 50
  |||
1  GGVPGAIPGGVPGGVFFPGAGLGGALGGGALGPGGKPLKPVPGGLAGAGLG 50
  |||
51  AGLGAFPAVTFPGALVPGGVADAAAAYKAAKAGAGLGGVPGVGGVGVSAG 100
  |||
51  AGLGAFPAVTFPGALVPGGVADAAAAYKAAKAGAGLGGVPGVGGVGVSAG 100
  |||
101 AVVPQPGAGVKPGKVPVGLPGVYPGGVLPGARFPGVGLPGVPTGAGVK 150
  |||
101 AVVPQPGAGVKPGKVPVGLPGVYPGGVLPGARFPGVGLPGVPTGAGVK 150
  |||
151 PKAPGVGGAFAGIPGVGPFGGPQPGVPLGYPIKAPKLPGGYGLPYTTGKL 200
  |||
151 PKAPGVGGAFAGIPGVGPFGGPQPGVPLGYPIKAPKLPGGYGLPYTTGKL 200
  |||
201 PYGYGPGGVAGAAGKAGYPTGTGVGPQAAAAAATAAAKFGAGAAGVLP 250
  |||
201 PYGYGPGGVAGAAGKAGYPTGTGVGPQAAAAAATAAAKFGAGAAGVLP 250
  |||
251 VGGAGVPGVPGAIPGIGGIAGVGTAAAAAATAAAKAAKYGAAGLVPG 300
  |||
251 VGGAGVPGVPGAIPGIGGIAGVGTAAAAAATAAAKAAKYGAAGLVPG 300
  |||
301 PGFGPGVVGVPAGVPGVPGVPGAGIPVVPAGIPGAAPGVVSPEAAKA 350
  |||
301 PGFGPGVVGVPAGVPGVPGVPGAGIPVVPAGIPGAAPGVVSPEAAKA 350
  |||
351 AAKAAKYGARPGVGVGGIPTYGVGAGGFPFGVGVGGIPGVAGVPSVGGV 400
  |||
351 AAKAAKYGARPGVGVGGIPTYGVGAGGFPFGVGVGGIPGVAGVPSVGGV 400
  |||
401 PGVGGVPGVGISPEAQAAAAAATAKYGVGTAAAAAATAAAQAQGLVPG 450
  |||
401 PGVGGVPGVGISPEAQAAAAAATAKYGVGTAAAAAATAAAQAQGLVPG 450
  |||
451 VGVAPGVGVAPGVGVAPGVGLAPGVGVAPGVGVAPGVGVAPGIGPGGVAA 500
  |||
451 VGVAPGVGVAPGVGVAPGVGLAPGVGVAPGVGVAPGVGVAPGIGPGGVAA 500
  |||
501 AAKSAAKVAQAQLRAAAGLGAGIPGLGVGVGPGLGVGAGVPGLGVGAG 550
  |||
501 AAKSAAKVAQAQLRAAAGLGAGIPGLGVGVGPGLGVGAGVPGLGVGAG 550
  |||
551 VPGFGAGADEGVRRSLSPELREGDPSSSQHLPSTPSSPRVPGALAAKAA 600
  |||
551 VPGFGA.....VPGALAAKAA 567
  |||
601 KYGAAPGVVLGGALGGVGPVGGVVGAGPAAAAAATAAAQAQGLV 650
  |||
568 KYGAAPGVVLGGALGGVGPVGGVVGAGPAAAAAATAAAQAQGLV 617
  |||
651 AAGLGGLGVGGIGVPGVGGIGGIPAAAAAATAKYGAAGLGGVLGGAGQFP 700
  |||
618 AAGLGGLGVGGIGVPGVGGIGGIPAAAAAATAKYGAAGLGGVLGGAGQFP 667
  |||
701 LGGVAARPFGLSPIFPGGACLGKACGRKK 731
  |||
668 LGGVAARPFGLSPIFPGGACLGKACGRKK 698
  |||

```

Figure 3

SHELDelta.modified coding sequence and inferred polypeptide

```

1 ATGGGTGGCGTTCCGGGTGCTGTTCGGGGTGGCGTTCCGGGTGGTGTATT 50
  |||
1 MetGlyGlyValProGlyAlaValProGlyGlyValProGlyGlyValPh 17
51 CTACCCAGGCGCGGGTTTCGGTGTCTTCGGGGTGGCGTTGCAGACGCG 100
  |||
18 eTyrProGlyAlaGlyPheGlyAlaValProGlyGlyValAlaAspAla 34
101 CTGCTGCGTACAAAGCGGCAAGGCAGGTGCGGGTCTGGGCGGGGTACCA 150
  |||
35 laAlaAlaTyrTyrAlaAlaLysAlaGlyAlaGlyLeuGlyGlyValPro 50
151 GGTGTTGGCGGTCTGGGTGTATCTGCTGGCGCAGTTGTTCCGCAGCCGGG 200
  |||
51 GlyValGlyGlyLeuGlyValSerAlaGlyAlaValValProGlnProGl 67
201 TGCAGGTGTAAACCGGGCAAGTTCCAGGTGTTGGTCTGCCGGGCGTAT 250
  |||
68 yAlaGlyValLysProGlyLysValProGlyValGlyLeuProGlyValT 84
251 ACCCGGGTTTCGGTGTCTTCGGGGCGCGGTTTCCAGGTGTGGTGTAT 300
  |||
85 yrProGlyPheGlyAlaValProGlyAlaArgPheProGlyValGlyVal 100
301 CTGCCGGGCGTTCCGACCGGTGCAGGTGTTAAACCGAAGGCACCAGGTGT 350
  |||
101 LeuProGlyValProThrGlyAlaGlyValLysProLysAlaProGlyVa 117
351 AGGCGGGCGGTTCCGGGTATCCCGGGTGTGGCCCGTTCCGGTGGTCCGC 400
  |||
118 lGlyGlyAlaPheAlaGlyIleProGlyValGlyProPheGlyGlyProG 134
401 AGCCAGGCGTTCGCTGGGTATCCCGATCAAGCGCCGAAGCTTCCAGGT 450
  |||
135 lnProGlyValProLeuGlyTyrProIleLysAlaProLysLeuProGly 150
451 GGCTACGGTCTGCGGTACACCACCGGTAAACTGCCGTACGGCTACGGTCC 500
  |||
151 GlyTyrGlyLeuProTyrThrThrGlyLysLeuProTyrGlyTyrGlyPr 167
501 GGGTGGCGTAGCAGGTGCTGCGGGTAAAGCAGGCTACCCAAACGGTACTG 550
  |||
168 oGlyGlyValAlaGlyAlaAlaGlyLysAlaGlyTyrProThrGlyThrG 184
551 GTGTTGGTCCGCGAGGCTGCTGCGGCAGCTGCGGCGAAGGCAGCAGCAAA 600
  |||
185 lyValGlyProGlnAlaAlaAlaAlaAlaAlaAlaLysAlaAlaAlaLys 200
601 TTCGGGCGGGGTGCAGCGGGTTTCGGTGTCTTCGGGGCGTAGGTGGTGC 650
  |||
201 PheGlyAlaGlyAlaAlaGlyPheGlyAlaValProGlyValGlyGlyAl 217
651 TGGCGTTCCGGGTGTTCCAGGTGCGATCCCGGGCATCGGTGGTATCGCAG 700
  |||
218 aGlyValProGlyValProGlyAlaIleProGlyIleGlyGlyIleAlaG 234
701 GCGTAGGTACTCCGGCGCGCGCTGCGGCTGCGGCAGCTGCGGCGAAGCA 750
  |||
235 lyValGlyThrProAlaAlaAlaAlaAlaAlaAlaAlaAlaLysAla 250
751 GCTAAATACGTTGCGGCAGCAGGCCTGGTTCCGGGTGGTCCAGGCTTCGG 800
  |||
251 AlaLysTyrGlyAlaAlaAlaGlyLeuValProGlyGlyProGlyPheGl 267
801 TCCGGGTGTGTAGGCGTTCCGGGTTCGGTGTCTTCGGGGCGTAGGTG 850
  |||

```

Figure 4(1)



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268 yProGlyValValGlyValProGlyPheGlyAlaValProGlyValGlyV 284
851 TTCCAGGTGCGGGCATCCCGGTTGTACCGGGTGACGGTATCCCGGGCGCT 900
|||
285 alProGlyAlaGlyIleProValValProGlyAlaGlyIle ProGlyAla 300
|||
901 GCGGGTTTCGGTGTGTATCCCGGAAGCGGCAGCTAAGGCTGCTGCGAA 950
|||
301 AlaGlyPheGlyAlaValSerProGluAlaAlaAlaIysAlaAlaAlaIy 317
|||
951 AGCTGCGAAATACGGAGCTCGTCCGGGCGGTGGTGTGGTGGCATCCCGA 1000
|||
318 sAlaAlaIysTyrGlyAlaArgProGlyValGlyValGlyGlyIleProT 334
|||
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|||
335 hrTyrGlyValGlyAlaGlyGlyPheProGlyPheGlyValGlyValGly 350
|||
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|||
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|||
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|||
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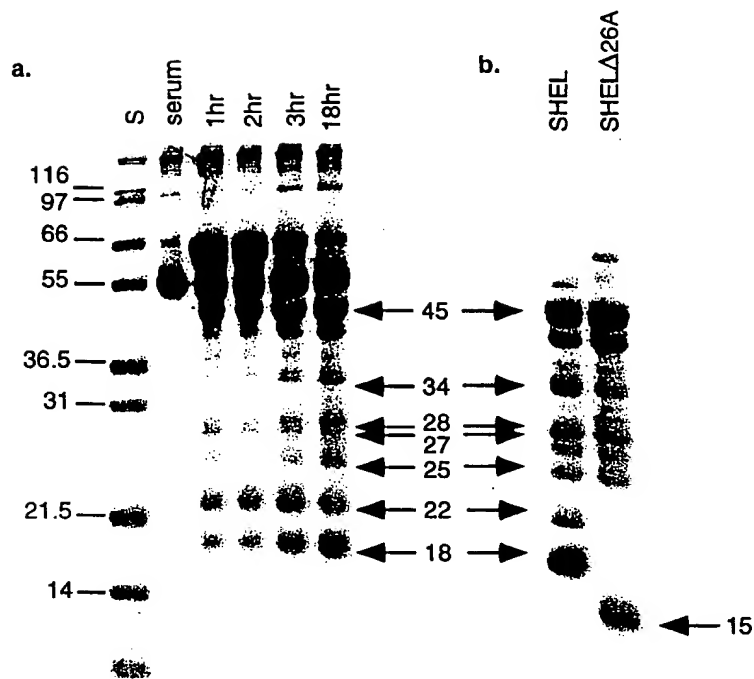
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601 GlyGlyIleProProAlaAlaAlaAlaIysAlaAlaIysTyrGlyAlaAl 617
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1851 AGGTCTGGGTGGCGTTCTGGGTGGTGTGGTTCAGTTCCCACTGGGCGGTG 1900
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618 aGlyLeuGlyGlyValLeuGlyGlyAlaGlyGlnPheProLeuGlyGlyV 634
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1901 TAGCGGCACGTCCGGGTTTCGGTCTGTCCCGATCTTCCAGGCGGTGCA 1950
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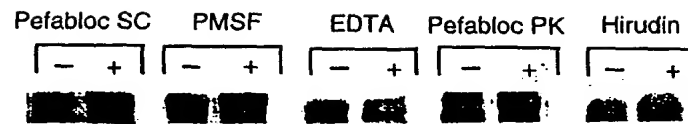
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Figure 4(3)



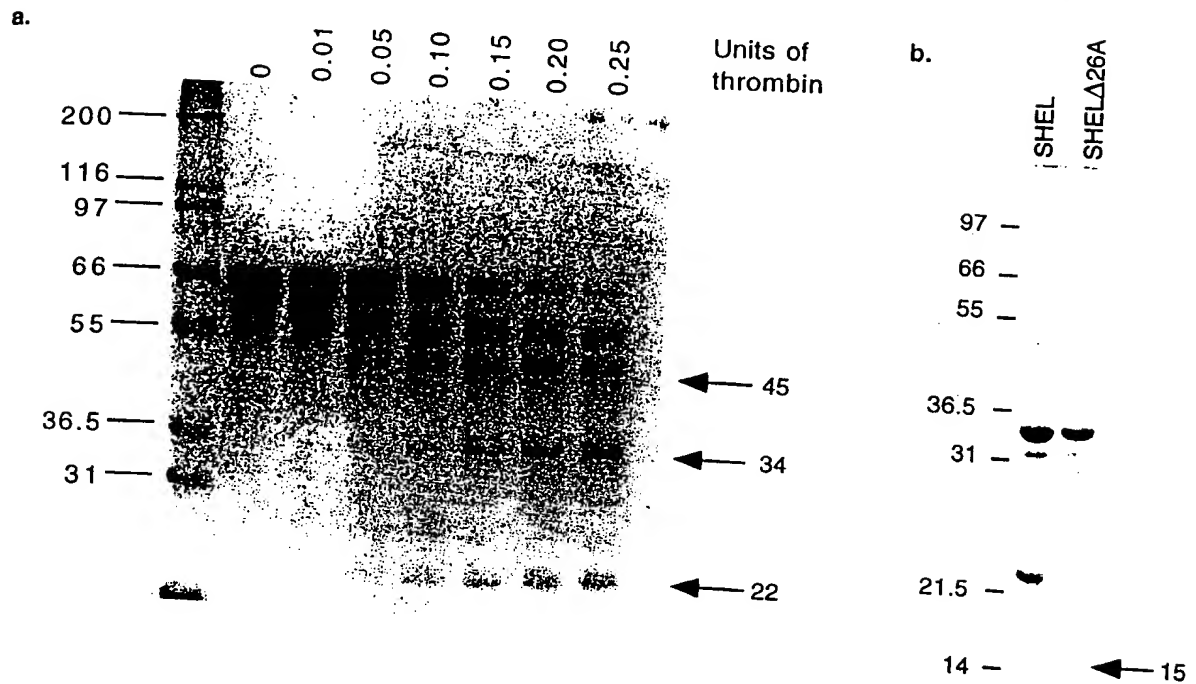
**Figure 5**

**Degradation of SHEL with serum.** **a.** After addition of serum to SHEL for 1, 2, 3 or 18hr, SHEL is fragmented into a number of distinct bands as seen by 10% SDS-PAGE. Products from overnight digestion are very similar to products present after one hour of digestion. **b.** SHEL and SHELΔ26A peptide fragments produced by serum digestion and purified by butanol solubilisation were analysed by 10% SDS-PAGE. A band at 15kDa appears with SHELΔ26A (arrow) in place of the 22 and 18kDa bands. Approximate sizes of fragments produced are shown in kDa. Size markers (S) are shown in kDa.



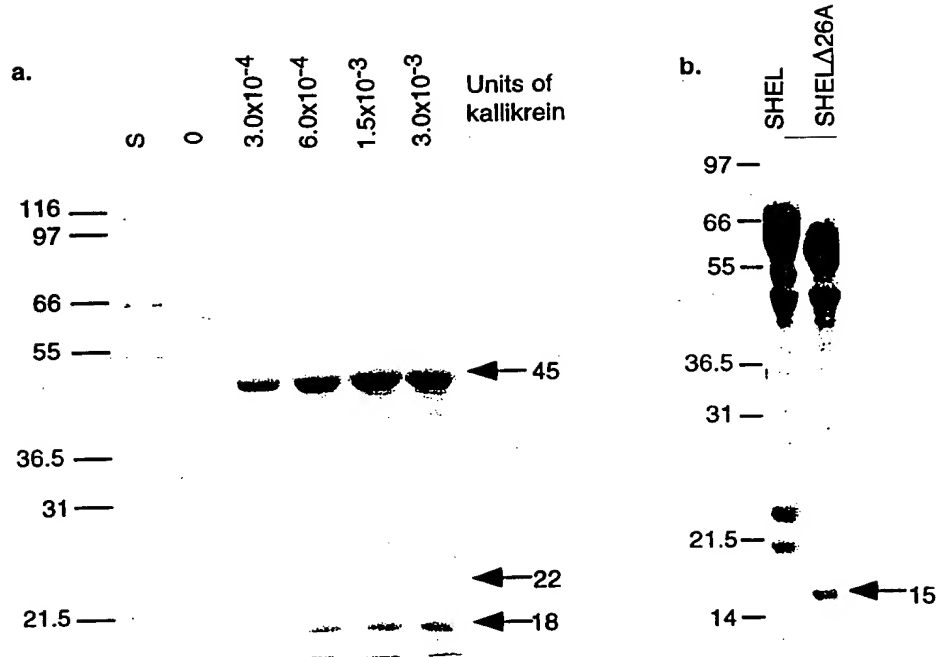
**Figure 6**

**Effect of protease inhibitors on serum degradation of SHEL.** The presence (+) or absence (-) of various protease inhibitors on the amount of full-length SHEL was analysed by 8% SDS-PAGE. Full-length SHEL is increased in the presence of Pefabloc SC (0.5mM), Pefabloc PK (50 $\mu$ M) and PMSF (5mM) compared with serum alone, while there is no noticeable effect in the presence of hirudin (1U). In contrast, the presence of EDTA results in a decrease in the amount of full-length SHEL.



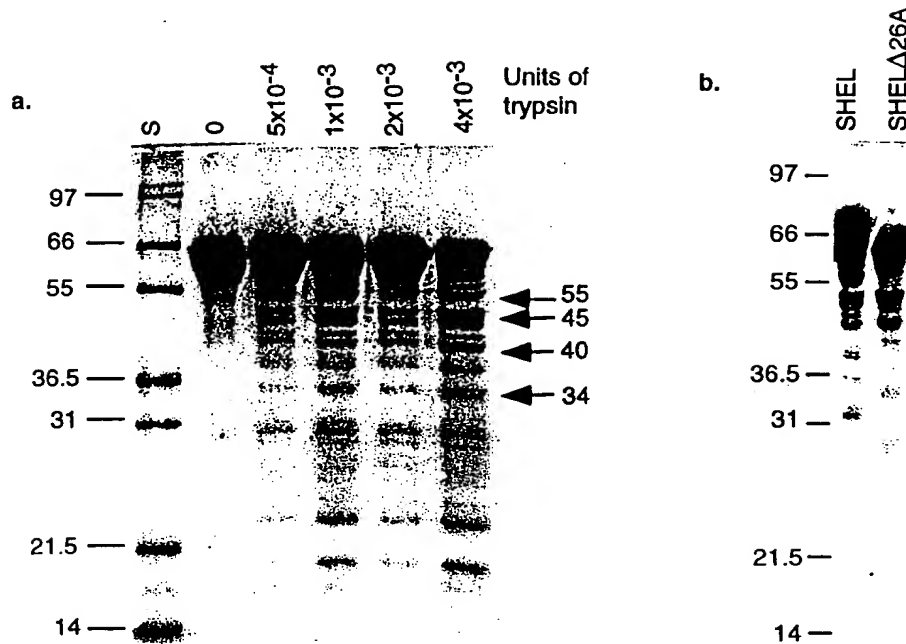
**Figure 7**

**Effect of thrombin on SHEL and SHELΔ26A.** a. Increasing amounts of thrombin were added to SHEL and analysed by 8% SDS-PAGE. Three major degradation products are seen estimated at 45, 34 and 22kDa, as well as a 13kDa fragment not seen in this gel. b. Effect of thrombin (1U) on degradation of SHELΔ26A compared with SHEL, analysed by 8% SDS-PAGE. A band at 15kDa (arrow) appears in place of the 22kDa band. Fragment sizes are estimated in kDa. Size markers (S) are shown in kDa.



**Figure 8**

**Effect of kallikrein on SHEL and SHELΔ26A.** a. Increasing concentrations of kallikrein were added to SHEL and analysed by 8% SDS-PAGE. Three major fragments are seen at 45, 22 and 18kDa. The 22kDa fragment disappears with higher concentrations or longer incubations with kallikrein. b. Effect of kallikrein ( $6 \times 10^{-4}$ U) on degradation of SHELΔ26A compared with SHEL, analysed by 8% SDS-PAGE. Only two fragments are seen with SHELΔ26A at 45 and 15kDa (arrow). Fragment sizes and size markers (S) are shown in kDa.



**Figure 9**

**Effect of bovine trypsin on SHEL and SHELΔ26A.** **a.** Increasing concentrations of bovine trypsin were added to SHEL and analysed by 10% SDS-PAGE. Dilute amounts of trypsin produce prominent bands at 50, 45, 40, 34, 31-25, 22 and 18kDa, similar to serum-produced peptides. Higher concentrations completely degrade SHEL (not shown). **b.** Effect of bovine trypsin ( $2 \times 10^{-3}$ U) on SHELΔ26A compared with SHEL, analysed by 10% SDS-PAGE. The overall pattern of fragments is the same as for SHEL but the size of the smaller fragments are all approximately 4kDa less. Fragment sizes and size markers (S) are shown in kDa.

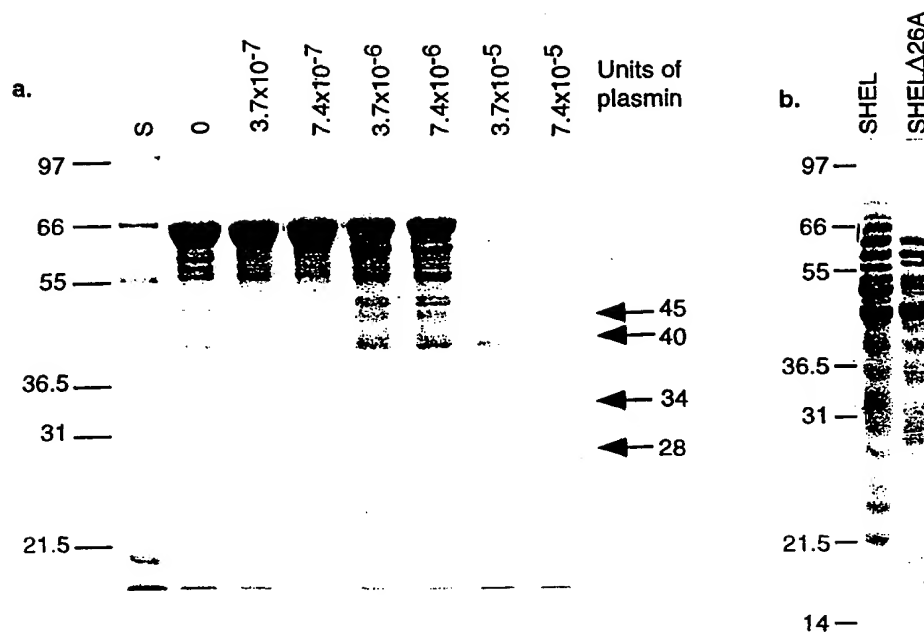


Figure 10

**Effect of plasmin on SHEL and SHEL $\Delta$ 26A.** a. Increasing concentrations of plasmin were added to SHEL and analysed by 10% SDS-PAGE. Dilute amounts of plasmin produce prominent bands at 50, 45, 40, 34, 28, 22 and 18kDa similar to serum-produced peptides. Higher concentrations of plasmin or longer incubations completely degrade SHEL (not shown). b. Effect of plasmin ( $7.4 \times 10^{-5}$ U) on SHEL $\Delta$ 26A compared with SHEL, analysed by 10% SDS-PAGE. The overall pattern is similar to SHEL but the smaller fragments are approximately 4kDa smaller. Fragment sizes and size markers (S) are shown in kDa.



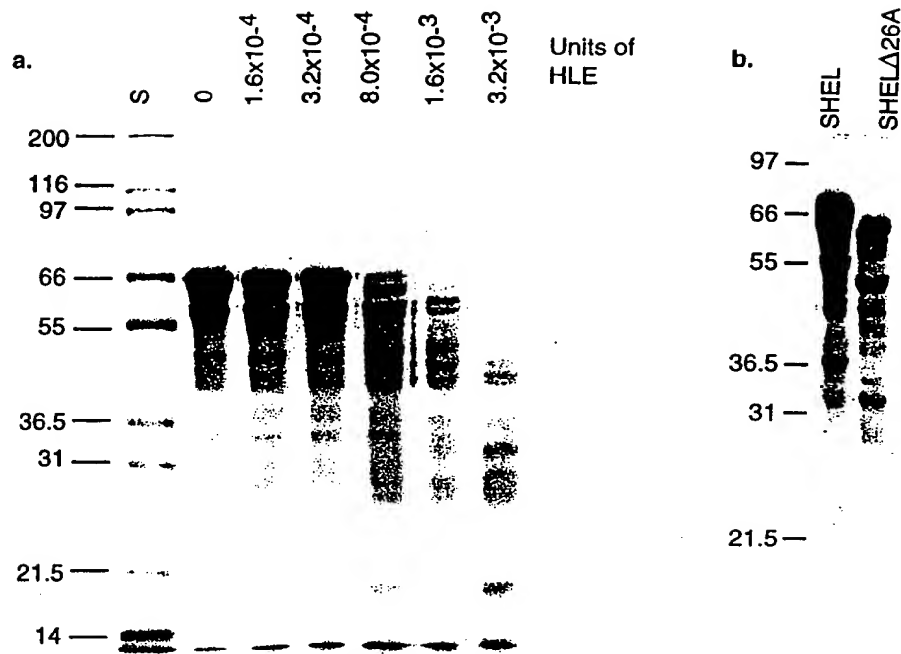


Figure 11

**Effect of HLE on SHEL and SHELΔ26A.** Increasing concentrations of HLE were added to SHEL and analysed by 10% SDS-PAGE. Degradation was extensive but prominent sharp fragments were identified at 32 and 18kDa with the other bands being diffuse. **b.** Effect of HLE ( $1.6 \times 10^{-3}$ U) on SHELΔ26A compared with SHEL, analysed by 10% SDS-PAGE. A very similar profile to that of SHEL is seen but fragments are uniformly 4kDa smaller. Fragment sizes and size markers (S) are shown in kDa.

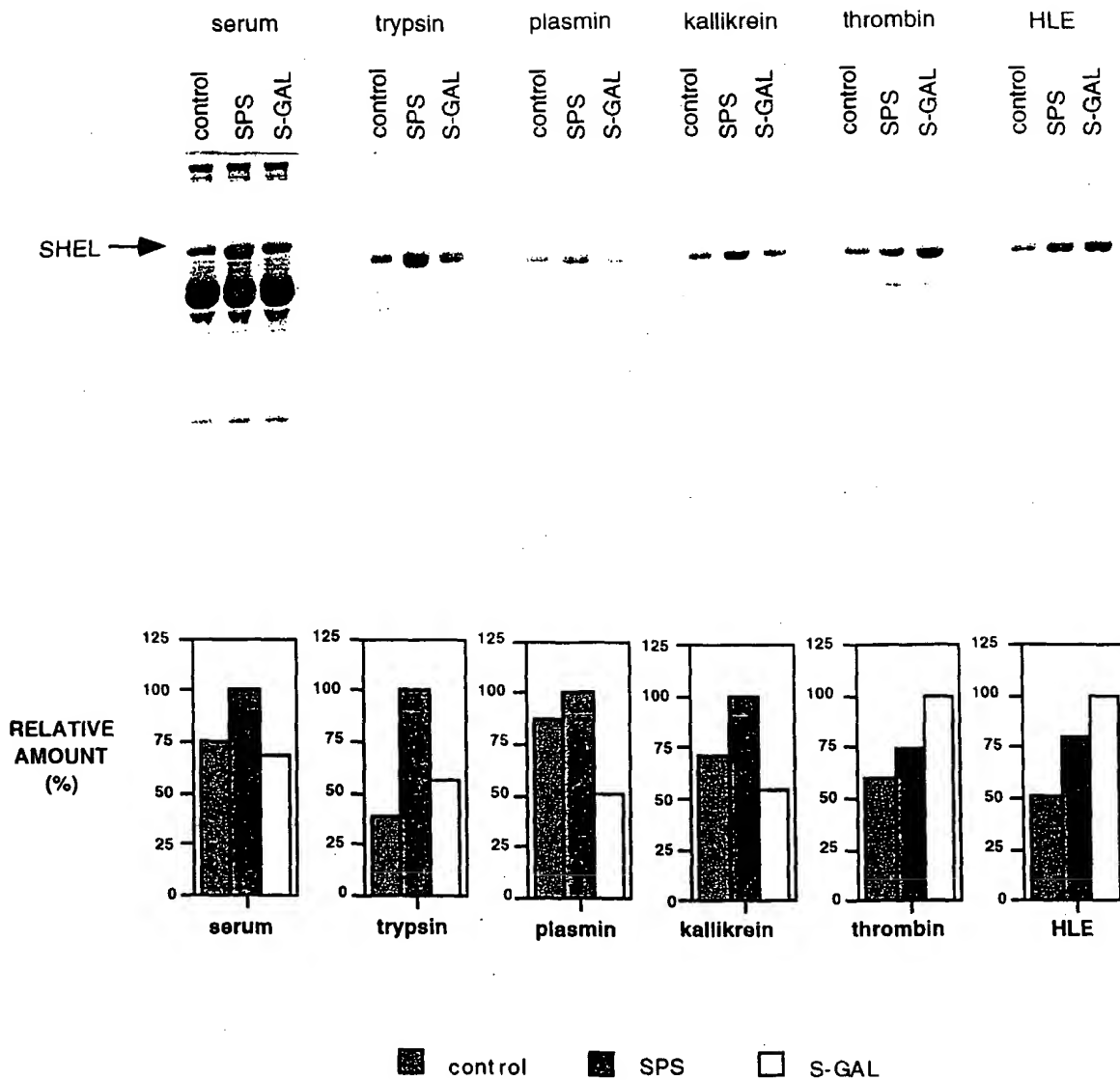


Fig. 12

**Typical effect of S-GAL and the SPS-peptide on degradation of SHEL.** Addition of SPS-peptide or S-GAL to SHEL reactions containing proteases was examined by 10% SDS-PAGE. The conditions used are serum, 1/2 dilution 20min; trypsin 20min; plasmin  $1.5 \times 10^{-5}$ U 20min; kallikrein  $15 \times 10^{-4}$ U 40min; thrombin 0.1U 20min; HLE 70min. Thrombin and kallikrein were used with a 200:1 peptide:SHEL ratio while the others were used with a 100:1 ratio. Gels were scanned by densitometry and the relative amount of each full-length SHEL band is shown in a histogram. In each case, the presence of SPS-peptide resulted in more full-length SHEL remaining than in control reactions, while the presence of S-GAL also resulted in more full-length SHEL in the presence of thrombin and HLE only.

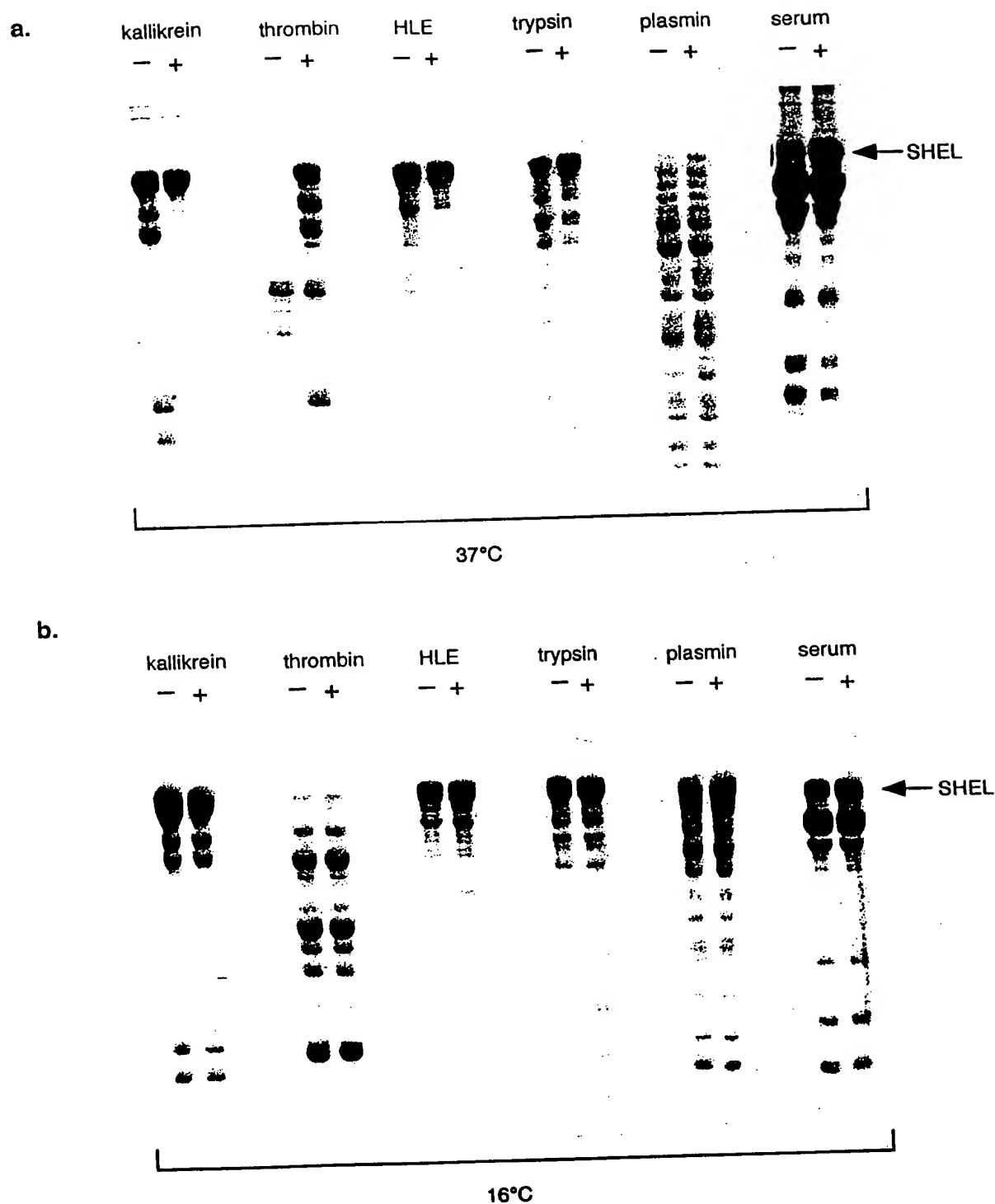


Fig. 13

**Effect of coacervation on the degradation of SHEL by proteases.** a. SHEL degradation in the presence of NaCl conducive to coacervation of SHEL at 37°C (+) was compared to SHEL which did not coacervate at 37°C (-). b. Control reactions in the presence (+) and absence (-) of NaCl were performed at 16°C. Significant protection from kallikrein and thrombin proteolysis is seen when SHEL is coacervated. Protection is also seen from serum, trypsin and HLE while none is seen with plasmin. At 16°C all of the proteases degraded SHEL to a similar extent in both the presence and absence of NaCl.

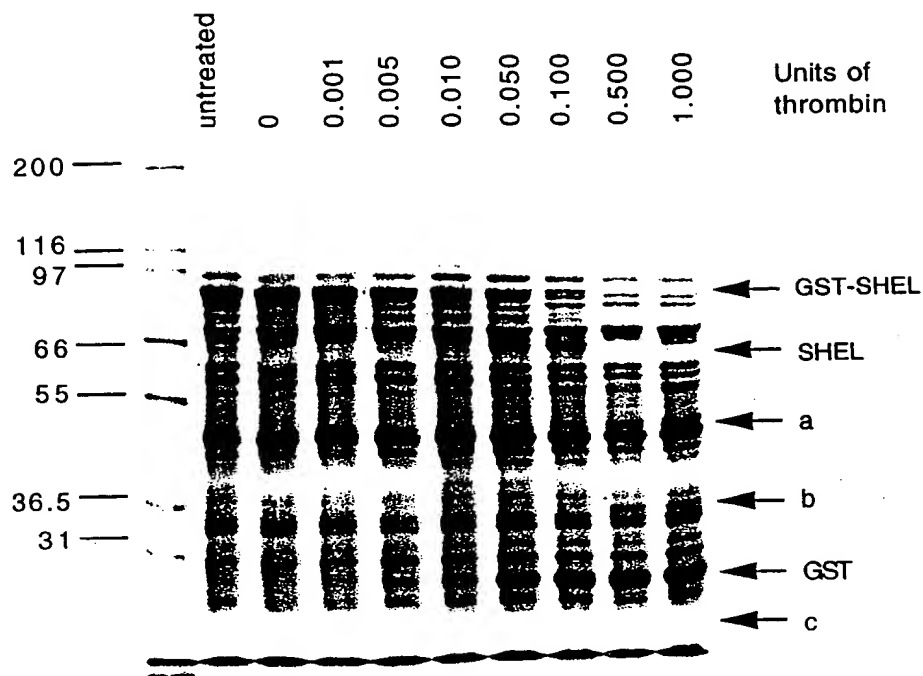


Fig. 14

**Thrombin cleavage of soluble cell lysate containing GST-SHEL.** Increasing amounts of thrombin (indicated in units) were added to soluble cell lysate and analysed by 8% SDS-PAGE. GST can be clearly seen at approximately 26kDa with thrombin concentrations above 0.01U while GST-SHEL decreases. SHEL, at approximately 64kDa, can be discerned at intermediate thrombin concentrations of 0.05 and 0.1U. Increasing thrombin further results in removal of the SHEL band and the appearance of three smaller bands (a,b,c) at approximately 45, 34 and 22kDa.

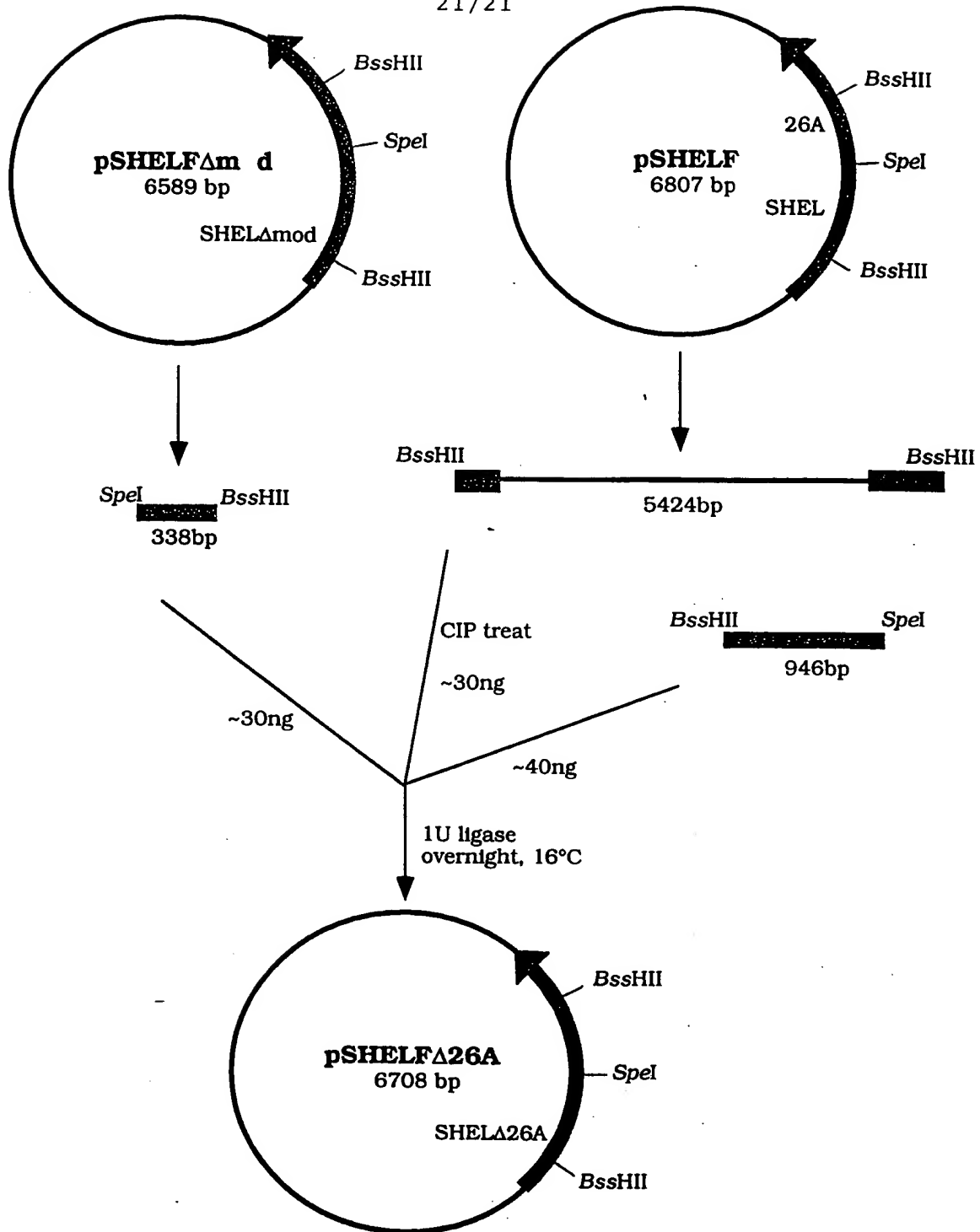


Fig. 15

**Construction scheme for pSHELFΔ26A.** pSHELF and the aberrant pSHELFΔmod were both digested with SpeI and BssHII. BssHII cuts both plasmids twice and SpeI once resulting in three fragments. The 5424 and 946bp fragments from pSHELF and the small 338bp fragment from pSHELFΔmod were purified from agarose gels. The 5424bp fragment was CIP treated to reduce recircularisation and the three fragments ligated overnight at 16°C using DNA ligase. The final product, pSHELFΔ26A contained the desired deletion of exon 26A from the SHEL gene with no other mutations.

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